UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

FACULTAD DE BIOLOGÍA

ANTHROPOGENIC NITROGEN IMPACTS IN LITTORAL ECOSYSTEMS:

QUANTIFICATION WITH N STABLE ISOTOPES USING LONG-LIVING MACROALGAE

Memoria que presenta

Inés González Viana

para optar al grado de Doctora en Biología

Fdo. Inés González Viana

Santiago de Compostela, Noviembre 2014

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Dr. **Antonio Bode Riestra**, Investigador en el Centro Oceanográfico de A Coruña del Instituto Español de Oceanografía,

CERTIFICA

Que la presente memoria titulada "**Anthropogenic nitrogen impacts in littoral ecosystems: Quantification with N stable isotopes using long-living macroalgae**", presentada por Dña. **Inés González Viana** para optar al grado de Doctora en Biología, ha sido realizada bajo mi dirección, cumpliendo las condiciones exigidas para su presentación, la cual autorizo.

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Fdo. Dr. Alejo Carballeira Ocaña
Tutor de la Tesis Doctoral.

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Contents

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General Introduction

Anthropogenic N in coastal systems: The eutrophication process

Human populations have been traditionally associated with N pollution. Residual waters and human associated activities, as agriculture and cattle industry, are linked to an excess of N inputs. This human-derived nitrogen wastes are mainly in forms as ammonium (NH_4^+), ammonia (NH_3), nitrate (NO_3^-) or urea, and end in the aquifers, rivers and finally in coastal waters.

It was in the 20th century, when humans began to have an enormous impact on the global nitrogen cycle by developing two key processes: The invention of the Haber-Bosch process and the increasing combustion of fossil fuels. The first process allows a reaction that is naturally restricted to a limited group of microorganisms, which converts the most common molecule that is also virtually inert, the gaseous nitrogen (N_2) into NH_3 (Heaton 1986). This process allowed an unlimited production of reactive nitrogen (N_r). The N_r includes inorganic reduced forms (as NH_3 or NH_4^+), inorganic oxidized forms (as NO_x, HNO_3, N_2O, NO_3^- or NO_2^-) and organic compounds (as urea, amino acids or nucleic acids). This process has helped to implant new agricultural practices, increasing their use as crop demands rise due to the increasing human population. This, together with the combustion of fossil fuels (Galloway and Cowling 2002), which mainly produces NO_x and N_2O, has led to a rapid increase of N_r, doubling the flow of available nitrogen and altering the nitrogen cycle at local and global scales (Tear 2013), as it produces an imbalance between denitrification processes and inputs.

An important fraction of the anthropogenically created N_r distributes into marine areas, especially in the coast, as these systems integrate the varying influence of both the land and the ocean (Bode et al. 2011b) and they are the final deposit of many contaminants (Prego et al. 2008). Moreover anthropogenic nitrogen loads have been closely linked to population densities in coastal watersheds (Nixon 1995) which continue to increase. In coastal marine systems, nitrogen has typically being the principal limiting element, as demonstrated by field observations and experiments (Valiela 1995). So the nitrogen content of any effluent entering coastal waters is of considerable importance for the management of the coastal zone, since it controls the

level of primary production of both phytoplankton and macroalgae (Valiela 1995). Fertility of coastal waters is often controlled by N inputs, provided either internally by regeneration of pre-existing N and biologically-fixed atmospheric N, or supplied externally as a combination of mostly anthropogenic N sources delivered via surface runoff, sub-surface groundwater or atmospheric deposition (Sigman 2009). However, there are also naturally occurring processes, as upwelling, that fertilize coastal areas worldwide (Valiela 1995).

Nowadays, the main consequence of the increasing inorganic nutrients inputs in coastal areas are the eutrophication processes (Nixon 1995). Eutrophication may be defined as the enhanced supply rate of organic matter into the ecosystem due to increased primary productivity (Nixon 1995). This process was not a major concern in open coastal areas, characterized by large dilution and water exchanges capacities. But the increasing N inputs due to human activities has created a disproportion between N inputs and denitrification processes, which is the major mechanism of fixed N loss from the ocean. Many authors have summarized the consequences of eutrophication processes in coastal areas, which include increases in algal production and growth, changes in primary-producers community, losses in water clarity, and oxygen depletion at ecosystem scale, which may finally alter the faunal community and fisheries (Paerl and Peihler 2008). The Baltic and Yellow Sea are two examples where eutrophication is a major concern (Bonsdorff et al. 1997, Teichberg et al. 2010, Liu et al. 2013). Therefore, nutrient pollution, especially nitrogen, and its link to eutrophication, has increasingly received a lot of attention by coastal managers worldwide but particularly at both sides of the Atlantic Ocean (Nixon and Fulweiler 2009).

Stable isotopes as tracers

Stable isotopes can be used as integrators and tracers of ecological processes at both naturally occurring levels and experimentally enriched abundances (Robinson 2001). The distribution of nitrogen isotopes within marine ecosystems can provide a record of sources of N supporting biological production and the major pathways and mechanisms moving nitrogen through the biota (Heaton 1986, Peterson and Fry 1987, Montoya 2008) providing ecological information across a range of spatio-temporal scales (Lepoint et al. 2004).

Fundamentals: Notation. Fractionation

The use of stable isotopes as tracers is based on the fact that N has two stable isotopes, a light isotope, ^{14}N, and a heavier isotope, ^{15}N, which occur in a constant proportion in the atmosphere, 99.635% and 0.365% respectively (Nier 1950). Natural isotopic compositions are then reported relative to the atmospheric nitrogen (air), the internationally accepted standard. The air was selected as the standard as the relation between both isotopes in this compartment is considered to be constant worldwide. As the nitrogen isotopic differences between materials are small, this relation is expressed in parts per thousand deviation from that standard by δ (delta) notation in units per mil (‰):

$$\delta^{15}N\ (‰) = (R_{sample}/R_{standard}-1) \times 10^3$$

where R is $\%^{15}N/(\%^{14}N+\%^{15}N)$.

In this notation, the $\delta^{15}N$ of atmospheric N_2 is 0‰. A positive $\delta^{15}N$ value indicates that the sample has more of the heavy isotope than does the standard whereas a negative $\delta^{15}N$ value indicates that the sample has less of the heavy isotope than the standard. The proportion of different substrata varies according to the different metabolic routes that a molecule follows, as the diverse reactions of the N cycle may discriminate (isotopic fractionation) against the heavy isotope (^{15}N). Isotopic fractionation is the disproportionate division of heavy and light isotopes between reaction substrates and products. It occurs because atomic masses and bound strengths are isotope dependent (Cravotta 1997).

Variations in ^{15}N in coastal environments: The N cycle

Significant variations in the natural abundance of ^{15}N in marine organisms were first documented in the 1950s (Hoering 1955), but the earliest studies focused on nitrogen isotopic abundance in marine systems were carried out since the mid-60s to study the variations in ^{15}N content of natural materials (Miyake and Wada 1967, Wada et al. 1975, Wada and Hattori 1976, Minagawa and Wada 1984, Owens 1987). The range of $\delta^{15}N$ values in marine systems extends from ~ -3 to 46‰ (Owens 1987). These studies contributed to understand the global distribution of nitrogen stable isotopes in the marine environment, which subsequently set the basis for the use of $\delta^{15}N$ as an indicator of the sources of nitrogen entering an ecosystem, and hence anthropogenic inputs (Sweeney and Kaplan 1980, Rau et al. 1981, Mariotti et al. 1984, Heaton 1986).

The marine nitrogen cycle involves multiple oxidation states of nitrogen and multiple pathways interconnect most of the biologically active pools (Montoya 2008). Physical, chemical, and biological processes that define the N cycle in marine environments discriminate (fractionate) between the two isotopes, leading to subtle but measurable differences in the $\delta^{15}N$ among different forms of nitrogen (Sigman et al. 2009). And fractionation, together with mixing processes, produces regular and characteristic isotope distributions (Fry 2006).

The major input of N in the ocean is by biological N_2 fixation. Fixed N adds combined nitrogen with a low $\delta^{15}N$ to the ocean, ~0‰, or ~0.6‰ in its dissolved form (Sigman et al. 2009). This biomass supplies new N to the dissolved fixed N pools (NH_4^+, NO_3^- and NO_2^-) in the surface and subsurface layers, which may be further available for other organisms. Other inputs to the marine environment in coastal areas include atmospheric precipitation and deposition, terrestrial runoff or anthropogenic loadings, wich are discussed in the next section (Gruber 2008).

Subsequent processes control the ocean´s fixed N counteracting both the mass and isotopic effects of N fixation. Chemical reactions that can alter the $\delta^{15}N$ signature include nitrification, denitrification and volatilization (Fig. 1.1). Nitrification and denitrification are both biologically mediated. Generally biological activities use ^{14}N preferentially, resulting in an increased $\delta^{15}N$ value in the remaining nitrogen. Nitrification that converts ammonium to nitrite and nitrate leaves residual ammonium enriched in ^{15}N (Mariotti et al. 1981). The inverse reaction (denitrification) produces ammonium isotopically lighter that the nitrate and nitrite used as substrata. The greater the denitrification via bacterial activity the higher the $\delta^{15}N$ value of the remaining substrata. This results in an increase of the $\delta^{15}N$ of the major pool of oceanic combined nitrogen, NO_3^- ($\delta^{15}N$ ~5-10‰, Fig. 1.1). It has been shown that this nitrate isotope signal of denitrification can be transported out of the region of denitrification by ocean circulation, as upwelling processes (Sigman et al. 2009). As a result of these reactions, mean NO_3^- values of the deep ocean average around 5‰ (Liu and Kaplan 1989). Remineralization of N from organic matter simply redistributes nitrogen among the different components of the bulk field of particulate organic matter but does not appear to have a significant impact on the distribution of isotopes in the upper water column (Montoya 2008). These internal cycling fluxes are neither sources nor sinks of fixed N but affect the distributions of N species and isotopes in the ocean (Gruber 2008).

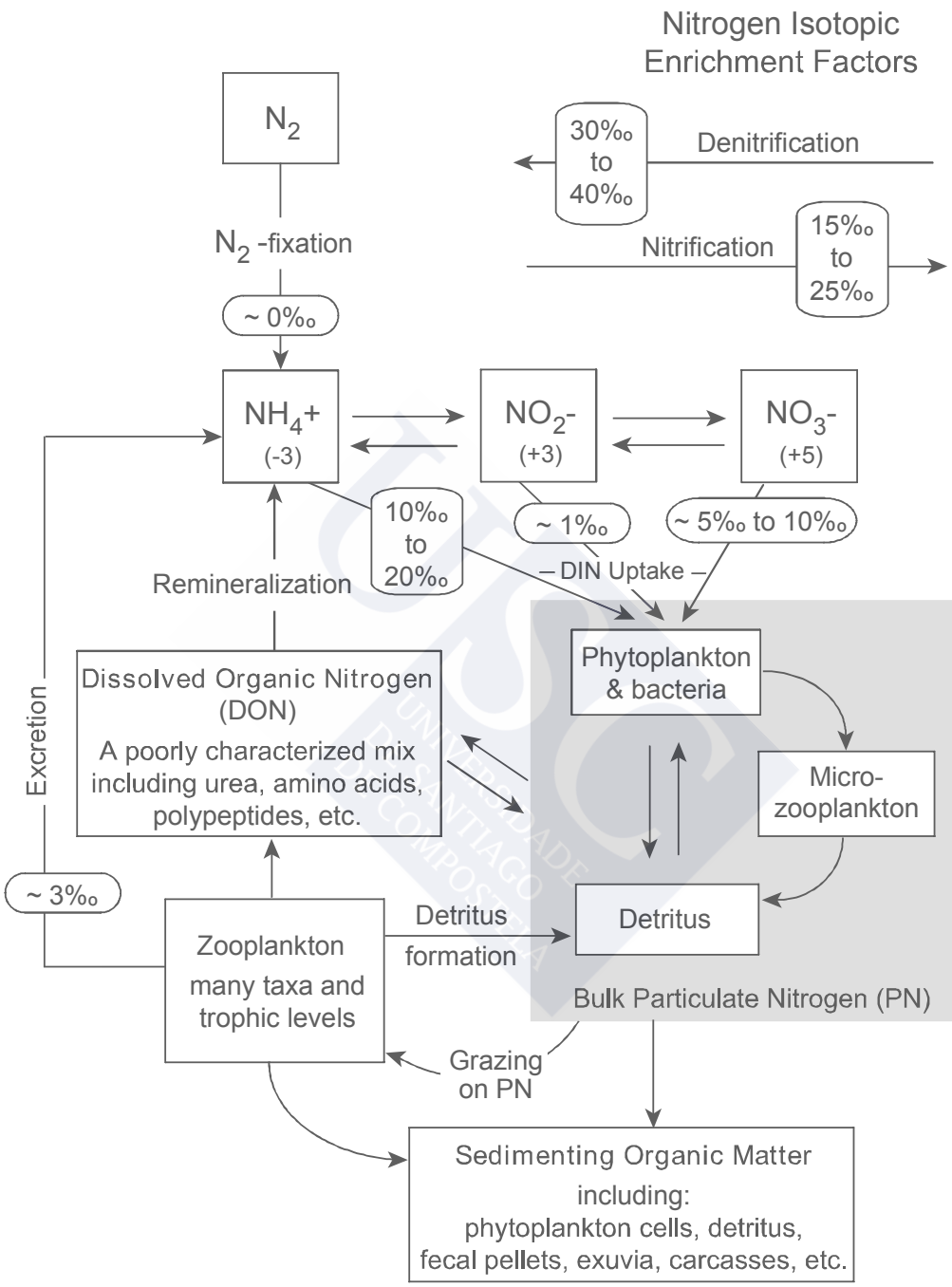

Figure 1.1. Schematic overview of the marine nitrogen cycle and the magnitude of isotopic fractionation associated with various reactions. Each box represents one major functional pool of nitrogen in the ocean. Arrows depict the major biological transformations of nitrogen. Typical values of the isotopic enrichment factor (ε) are given for reactions that have been characterized isotopically (adapted from Montoya 2007).

Tracing anthropogenic inputs

Anthropogenic loads often increase the $\delta^{15}N$ of dissolved inorganic nitrogen (DIN) of a system because they encourage denitrification (Mariotti et al. 1981, 1984; Sigman et al. 2009). The main sources of anthropogenic inputs are nitrogenous fertilizers, and animal or sewage wastes (Heaton 1986). DIN derived from sewage and farm wastes may be significantly enriched in ^{15}N as a result of volatilization and microbial processing of the nitrogen in solution (Sweeney and Kaplan 1980, Rau et al. 1981, Heaton 1986, Van Dover et al. 1992). Animal waste has a high ammonium-N component; the volatilization of ammonia gas causes the loss of ^{14}N with a resulting enrichment of ^{15}N in the remaining nitrogen, which is converted to nitrate.

In contrast to these anthropogenic sources, agricultural activity generally results in low $\delta^{15}N$ values in nitrate of the adjacent surface waters. Fertilizers have a wide range of values because of the variety of source materials used to create them. Ammonium-based fertilizers can be converted to nitrate by the bacterially mediated process of nitrification (Kendall and Caldwell 1998). The volatile loss of ammonia from organic and inorganic fertilizers, and denitrification in soils leads to higher $\delta^{15}N\text{-}NO_3^-$ values of nitrate in rivers receiving large amounts of N from agriculture, compared with natural watersheds (Flipse and Bonner 1985, Mayer et al. 2002). In contrast, ammonium fertilizers, which are produced by fixation of atmospheric N (by the Haber-Bosch process), show small differences in $\delta^{15}N$ as a result of small fractionation during subsequent processing of the fixed N (Flipse and Bonner 1985). All these pollutant sources in the environment have characteristic isotopic composition ranges (Fig. 1.2). These isotopic signatures can also vary while nutrients move from terrestrial to aquatic systems and along watersheds, and the N cycle may increase the $\delta^{15}N$ values through subsequent nitrification, denitrification or volatilization processes (Bedard-Haughn et al.2003). Therefore, $\delta^{15}N$-DIN can be used to interpret the source of environmental contamination (Heaton 1986, Lindau et al. 1989, Aravena et al. 1993).

Macroalgae as indicators of N sources: N stable isotopes biomonitoring

Even though $\delta^{15}N$-DIN values have been widely used for assessing anthropogenic N loadings (Kendall 1998, Mayer et al. 2002, Deutsch et al. 2005), in the last years several limitations of this method for monitoring purposes have arisen. While the determination

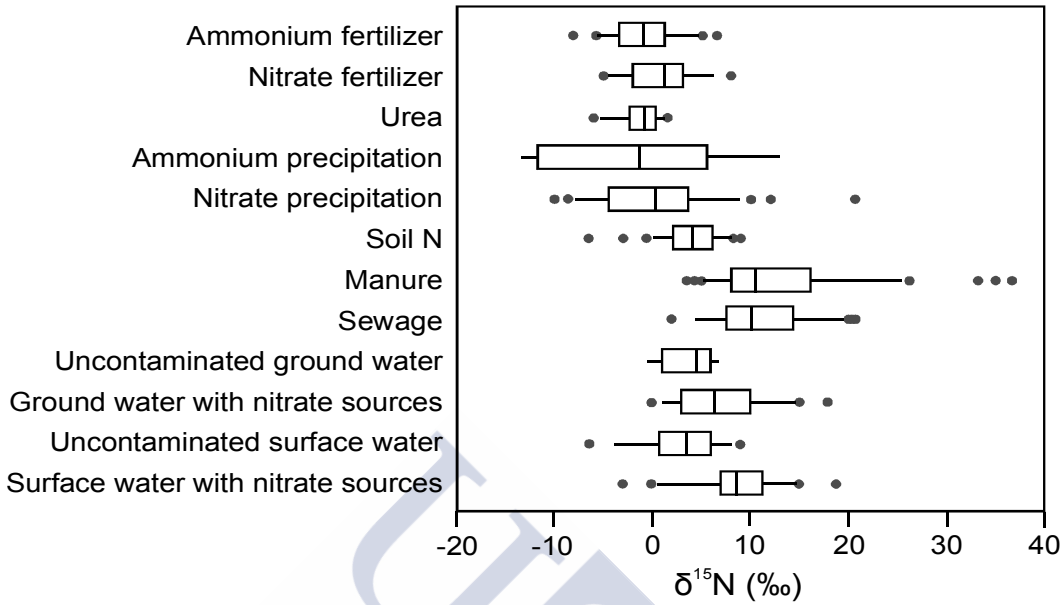

Figure 1.2. Box plots of $\delta^{15}N$ values of NO_3^- from various sources and sinks. Box plots illustrate the 25th, 50th and 75th percentiles; the whiskers indicate the 10th and 90th percentiles; and the circles represent outliers (adapted from Xue et al. 2009).

of isotopic values of dissolved nutrients help to identify nutrient sources, there exists high variability due to tidal exchanges and transient influx of nutrient concentrations in some coastal areas (Valiela et al. 1997). So given that nutrients may vary as a result of a wide range of factors, it is difficult to clearly identify all sources from instantaneous water measurements of isotopic values of DIN components. Moreover, stable isotope measurements on dissolved nutrients may not reflect what is actually available to primary producers, as saturation uptake kinetics or differential growth periods along the year have been observed (Lyngby 1990). Partly as a result of these limitations or the possibly higher effort of $\delta^{15}N$-DIN analysis compared to determinations in biota, increasing attention has been given to macroalgae as biomonitors of changes in the nutrient loads of coastal waters.

Nearly all transformations of the N cycle are undertaken by marine organisms as part of their metabolism, either to obtain nitrogen to synthesize structural components, or to gain energy for growth (Gruber 2008). This internal cycling may result in different nitrogen reservoirs of intermediary biota with also characteristic isotopic signatures (Owens 1987). From an isotopic standpoint, all of the different sources of N discussed in the previous section may also alter the baseline $\delta^{15}N$ of

the marine biota. Theoretically, direct detection of wastewater N in biota should provide a means to identify contributions of N to estuarine food webs before increased N availability leads to visible changes of population and community (McClelland and Valiela 1998b).

Several studies have exploited the characteristic isotopic signatures of anthropogenic N sources in macroalgae (McClelland and Valiela 1998b, Costanzo et al. 2001, Gartner et al. 2002, Alquezar et al. 2013). The advantages of these primary producers are that they are easy to identify, sample and process, and they are widely distributed. They accumulate dissolved substances in proportion to ambient bioavailability, offering time-integrated measures of the exposure of the biomonitor to sources over a previous time period (Rainbow and Phillips 1993). As they are sessile, they can integrate water variations of a particular site. Attending to nutrition strategies, different groups can be distinguished, as ephemeral and perennial species (Martínez et al. 2012).

Among ephemeral or fast-growing species, several green and red macroalgae can be included, as *Ulva* sp. All of them have in common their high nitrogen requirements (Pedersen and Borum 1997). This high nitrogen demands are associated with their high growth rates and, to a lesser extent, to their high tissue-N content (Hanisak 1983). Their capacity for N storage is also more limited than in perennial species, resulting in short-term storage that is gained through surge uptake. In contrast, perennial species, as fucoid macroalgae, are long-living species, and due to their slower growth rates, their N demand is also lower. This group of species has, in addition, higher capacity for utilizing internal N stores to cover their requirements for growth at low external N availability than the ephemeral macroalgae (Pedersen and Borum 1996). These differences in physiology and ecology have been shown to make a difference between N metabolism and requirements of both groups (Lobban and Harrison 1994). Extensively, these differences might influence the bioavailable N isotopic fraction reflected by the different species. Therefore, assessment of the relation of different species and stable isotopes needs to be separately tested.

Fast-growing macroalgae have received large attention in order to find the linkages between anthropogenic sources of N and its influence on enhancing primary production (macroalgal blooms). Therefore a number of studies in the last years have focused on understanding how these opportunistic macroalgae and stable

isotopes variability are related (Naldi and Wheeler 2002, Rogers 2003, Barile 2004, Cohen and Fong 2004, Lapointe et al. 2005, Dailer et al. 2010, Barr et al. 2013). Due to their seasonal living-strategy, these macroalgae may not be found year-round at some locations, so they might not be useful when studying the overall status of a particular site. In contrast, long-living macroalgae may not reflect sporadic nutrient changes, but may be suitable for long-term studies. The suitability of fucoid macroalgae for monitoring N loading have been also tested in several locations and under different N sources (Table 1.1).

Despite the number of studies using $\delta^{15}N$ values in brown macroalgae for monitoring purposes, there are almost no studies examining sources of variability affecting the isotopic values and net fractionation, especially in fucoid species (Umezawa et al. 2007, García-Sanz 2009). Therefore, the use of stable isotopes on these macroalgae to detect N loading has required some assumptions related to their ecology, physiology and fractionation processes. As shown by studies in other brown algae, species-specific chemical and physiological analysis, and understanding of their individual responses to various environmental factors are needed to interpret algal isotopic signatures (Umezawa et al. 2007).

Among fucoid macroalgae, *Fucus* spp. and *Ascophyllum nodosum* are widely distributed at both sides of the Atlantic Ocean, from the White Sea to the Iberian Peninsula at the East coast, and from around 77° N to New Jersey on the western side (Baardseth 1970). They show apical growth, so the growing tip is the part of the frond that has been traditionally considered in monitoring studies (Table 1.1). In these studies the current trophic status of different environments were characterized by the macroalgal $\delta^{15}N$.

Other interesting feature of their apical growth is the use of these species in retrospective studies. The $\delta^{15}N$ values of different segments along the frond can be related with past growing periods, and hence with ambient N status during those periods, if their growth rate is known. Savage and Elmgren (2004) estimated the year of growth of different sections of the thallus of *Fucus vesiculosus* from the number of bifurcations. However, specific studies showed large local variability and thus the inconsistent relationship between the number of bifurcations and growth in this species (Knight and Parke 1950). In contrast, *A. nodosum* individuals produce annual gas bladders that have been used in other studies to delimitate different exposure periods to different contaminants (Heldal and Sjøtun 2010);

Table 1.1. Overview of studies using $\delta^{15}N$ values in *Fucus* spp. and *A. nodosum* to track anthropogenic inputs in coastal systems.

Species	$\delta^{15}N$	$\delta^{15}N$ source	N source	Location	Sampling period	Part of the tissue	n	Reference
Fucus spp.	1.27-10.41	-	fish farms (44-2,250 t $year^{-1}$)	Galicia, Spain	July 2008	tips (3 cm)	~30	Carballeira et al. 2013
Fucus spp.	5.6-5.9	-	reference sites	Galicia, Spain	July 2008	tips (3 cm)	~30	
F. vesiculosus	7.18-10.47	-	fish farm (2, 250 t $year^{-1}$)	Galicia, Spain	July 08, 09, 10	retrospective study	3	Carballeira et al. 2014
F. vesiculosus	4.75-6.77	-	reference site	Galicia, Spain	July 08, 09, 10	retrospective study	3	
A. nodosum	9.4	9.3	WWTP outfalls, septic systems	Oyster River, Great Bay, NH, USA	-	-	-	Cole et al. 2004
F. vesiculosus	5.6-7.9	3.7-9.0	63% agriculture, 24% forests, 7% urban area	Warnow River Estuary, Germany	Feb-March 2004	tips (1-1.5 mm)	5	Deutsch and Voss 2006
F. vesiculosus	7.3	6.7-9.7	63% agriculture, 24% forests, 7% urban area	Warnow River Estuary, Germany	May 2004	tips (1-1.5 mm)	5	
F. vesiculosus	11-13	-	< 15 km from treated sewage of 250,000 inhab	Himmerfjärden Bay, Sweden	May	tips	-	Hobbie et al. 1990
F. vesiculosus	4.6	-	at 35 km from treated sewage of 250,000 inhab	Himmerfjärden Bay, Sweden	May	tips	-	
F. spiralis	9.2	-	STP	Hope Island, Narragansett Bay, RI, USA	September 2006	-	1	Oczkowski et al. 2008
F. spiralis	8.7	-	STP	Dutch Island, Narragansett Bay, RI, USA	September 2006	-	1	
Fucus sp.	7.7	-	STP	Beavertail, Narragansett Bay, RI, USA	September 2006	-	2	
A. nodosum	9.8	-	STP	Whale Rock, Narragansett Bay, RI, USA	September 2006	-	1	
F. spiralis	8.4	-	STP	Whale Rock, Narragansett Bay, RI, USA	September 2006	-	2	
F. spiralis	9.6	-	STP	Gould Island, Narragansett Bay, RI, USA	September 2006	-	1	
A. nodosum	8.1	-	STP	Castle Hill, Narragansett Bay, RI, USA	September 2006	-	1	
F. distichus	7.9	-	STP	Castle Hill, Narragansett Bay, RI, USA	September 2006	-	1	
Fucus sp.	8.2	-	offshore influence	Block Island Great Salt Pong, RI, USA	September 2006	-	1	
F. vesiculosus	9.5±1		culture, pasture, STP	Marebes-Oléron Bay, France	Jan-May 2006	tips (2 cm)	3	Raimonet et al. 2013
F. vesiculosus	9.8±1.3	8.5±3.9	culture, pasture, STP	Marebes-Oléron Bay, France	Jan-May 2006	tips (2 cm)	3	
F. vesiculosus	6.4±2.3	7.4±0.8	reference oceanic influence	Marebes-Oléron Bay, France	Jan-May 2006	tips (2 cm)	3	
F. serratus	10.5±0.3		culture, pasture, STP	Marebes-Oléron Bay, France	Jan-May 2006	tips (2 cm)	3	
F. serratus	8±2.1		culture, pasture, STP	Marebes-Oléron Bay, France	Jan-May 2006	tips (2 cm)	3	
F. serratus	2.1±3.3	7.4±0.8	reference oceanic influence	Marebes-Oléron Bay, France	Jan-May 2006	tips (2 cm)	3	
F. vesiculosus	25.7±3.8	-	eutrophic tidal estuary	The Westerschelde Estuary, The Netherlands	Jan-Feb 1998	-	2	Riera et al. 2000
F. vesiculosus	6.3±0.2	-	tidal bay	The Oosterchelde Estuary, The Netherlands	Jan-Feb 1998	-	2	
F. vesiculosus	8-9	38	<4 km from STP	Himmerfjärden Bay, Sweden	May 1999	tips (~2 cm)	3	Savage and Elmgren 2004
F. vesiculosus	5-6	38	>4 km from STP	Himmerfjärden Bay, Sweden	May 1999	tips (~2 cm)	3	
F. vesiculosus	3-4	4	14 km (coastal reference station)	Himmerfjärden Bay, Sweden	May 1999	tips (~2 cm)	3	
F. vesiculosus	10.5-14	24-38	~1km	Himmerfjärden Bay, Sweden	May 2002	retrospective study	3	
F. vesiculosus	3.4-3.8	4	coastal reference station	Himmerfjärden Bay, Sweden	May 2002	retrospective study	3	
F. ceranoides	2.17-14.57	-	sewage, upwelling, agriculture	Galicia, Spain	July 1990-2007	tips (3 cm)	~30	Viana et al. 2011
F. spiralis	6.12-11.33	-	sewage, upwelling, agriculture	Galicia, Spain	July 1990-2007	tips (3 cm)	~30	
F. vesiculosus	3.96-11.23	-	sewage, upwelling, agriculture	Galicia, Spain	July 1990-2007	tips (3 cm)	~30	

although there is also some uncertainty in determining when the first gas bladder appears (Cousens 1984). Thus, the growth, number of bifurcations and the timing in the appearance of the gas bladders need to be quantitatively tested for a feasible use of *Fucus* spp. and *A. nodosum* fronds in punctual and retrospective studies. The lack of site-specific growth studies, determines the necessity of quantitative growth measurements in the areas of interest, especially due to the large type of habitats where these species live.

Apart from the species-specific growth rates, it is necessary to understand the N metabolism along the macroalgal thallus. The main assumption of retrospective studies is that only the growing tips of the thallus take up nitrogen and, therefore, the isotopic composition of a given section of the thallus would reflect the isotopic composition of the dissolved nitrogen in the surrounding water at the time of growth (Savage and Elmgren 2004). Therefore, some questions need to be tested to fully interpret the data obtained in these studies. First, Fucaceae do not have a specific transport tissue, but the pores of the sieve plates would enable a continuous system of cytoplasm for the translocation of materials longitudinally (Moss 1983). If nitrogen transport along the thallus exists, it would directly affect the retrospective identification of past nitrogen sources. Second, most studies assume that the isotopic composition of tissues does not change for at least several months, given that these species generally show low variability in $\delta^{15}N$ values at monthly time scales (Gartner et al. 2002) but no data of N-specific uptake and turnover rates are available for most Fucacean species.

As they are long-living perennial species, it is also likely that isotopic signatures in macroalgae may be confounded by a variety of other factors in the environment, as seasonal effects or responses to local factors. For instance, the coast of the southern distribution limit of these species is eutrophized by seasonal upwelling processes (Álvarez-Salgado et al. 1997), while the Baltic Sea is known to be mainly eutrophized by the increasing anthropogenic influence (Bonsdorff et al. 1997). If the sources of isotopic variability at large environmental or geographical scales are not known, it would be difficult to exploit the isotopic values of these species and relate it to anthropogenic pressures in regional-scale studies.

They also live in the intertidal area of a wide variety of environments; therefore they are subject to a wide spatial and seasonal variability of abiotic factors and sources (e.g. salinity, temperature). This variability might significantly influence isotopic values in their tissues, but whether the variability observed in some studies is due to the sources or to the N metabolism in macroalgae (i.e. net fractionation of all processes within macroalgal tissues) is not known. Simultaneous measures of both macroalgae and seawater need to be done to detect the reasons of this variability (Deutsch and Voss 2006).

HYPOTHESIS AND GENERAL OBJECTIVES

Taking into account the present knowledge on the aplication of $\delta^{15}N$ in Fucaceae for monitoring anthropogenic N inputs to the coastal zone, and the information that is lacking, the following hypothesis was established:

- The proportion of N stable isotopes in the structural tissues of long-living macroalgae reflects the different utilization of natural and anthropogenic sources of nitrogen at long-time scales (months, years).

To validate the former hypothesis, the following principal and partial objectives were established:

- To determine the relation between the natural abundance of N stable isotopes in macroalgae and the origin of the sources of available N.

    1. To determine the geographical variability of the isotopic composition of the macroalgae *Ascophyllum nodosum* and *Fucus* spp. in relation to natural (e.g. upwelling) and anthropogenic (e.g. urbanization) factors.
    2. To determine the growth rates of *A. nodosum* and *Fucus vesiculosus* for their possible application to retrospective studies of nitrogen inputs.
    3. To determine the permanence period of N in different parts of the macroalgal frond.
    4. To quantify the anthropogenic N impact on intertidal macroalgae.

GENERAL STRUCTURE OF THE PhD THESIS

The data of the present dissertation were gathered during the years 2010-2013 within the project "Anthropogenic nitrogen inputs to littoral ecosystems: basis for monitoring using stable isotopes" (ANILE; CTM2009-08396 and CTM2010.08804-E). The general aim of this project was a precise quantification of natural and anthropogenic nitrogen inputs to littoral ecosystems. The specific objectives were to obtain information on the variability in the concentrations of dissolved nitrogen forms, and determine the isotopic signatures in the main components of plankton and benthos compartments. The objectives of the present thesis were within the partial objectives of this project.

To fulfill the previously numbered objectives, the analysis of these data was divided in different chapters that are structured as follows:

In Chapter 2 the natural and anthropogenic variability of N stable isotopes in macroalgae (*F. vesiculosus* and *A. nodosum*) is analyzed along a biogeographic gradient in an upwelling area (NW Spain). The influence of the upwelling seasonal process along the coast is based on previous studies, which showed that the influence is weaker from south to north. The anthropogenic influence is estimated based on the population size of the urban nuclei.

The third and fourth chapters aim to study the growth rates of both target species (*F. vesiculosus* and *A. nodosum*) with the aim of using the resulting growth curves in retrospective studies. The appearance of gas bladders in *A. nodosum* is also studied together with other aspects of the ecology of both species.

In the fifth chapter different assumptions when using macroalgae (*F. vesiculosus* and *A. nodosum*) as retrospective biomonitors are tested. First, the study focused on elucidating the turnover times of different parts of the tissue under different experimental and natural sources of water. Second, the existence of uptake capacity of growing and non-growing parts of the frond was determined. In addition, the possible existence of long-distance transport of nitrate along the thallus was tested in both species.

The understanding of processes affecting the variability of $\delta^{15}N$ in the selected macroalgae gathered in previous chapters were applied in the sixth chapter. A

local study of the influence of different terrestrial (i.e. river discharge) and natural (i.e. upwelling) N sources to these macroalgae was done at a local scale. In this chapter, various spatial and temporal scales of variability are considered.

The seventh chapter presents a synthesis of the main results and discussion within this dissertation. A general discussion of the implications of our findings and some future prospects are also suggested.

Finally, the last chapter gathers the main conclusions of this PhD Thesis.

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*Stable nitrogen isotopes in coastal macroalgae: Geographic and anthropogenic variability**

Abstract

Growing human population adds to the natural nitrogen loads to coastal waters. Both anthropogenic and natural nitrogen is readily incorporated in new biomass, and these different nitrogen sources may be traced by the measurement of the ratio of stable nitrogen isotopes ($\delta^{15}N$). In this study $\delta^{15}N$ was determined in two species of macroalgae (*Ascophyllum nodosum* and *Fucus vesiculosus*), and in nitrate and ammonium to determine the relative importance of anthropogenic versus natural sources of nitrogen along the coast of NW Spain. Both algal species and nitrogen sources showed similar isotopic enrichment for a given site, but algal $\delta^{15}N$ was not related to either inorganic nitrogen concentrations or $\delta^{15}N$ in the water samples. The latter suggests that inorganic nitrogen inputs are variable and do not always leave an isotopic trace in macroalgae. However, a significant linear decrease in macroalgal $\delta^{15}N$ along the coast is consistent with the differential effect of upwelling. Besides this geographic variability, the influence of anthropogenic nitrogen sources is evidenced by higher $\delta^{15}N$ in macroalgae from rias and estuaries compared to those from open coastal areas and in areas with more than $15x10^3$ inhabitants in the watershed. These results indicate that, in contrast with other studies, macroalgal $\delta^{15}N$ is not simply related to either inorganic nitrogen concentrations or human population size but depends on other factors as the upwelling or the efficiency of local waste treatment systems.

KEYWORDS:

upwelling
wastewater
urban populations
biomonitors
Fucus
Ascophyllum

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Introduction

Coastal areas, particularly estuaries, have been subjected to increasing nitrogen loads due to the growing human population and its associated anthropogenic activities (e.g. agriculture, sewage). As a consequence of these activities, coastal ecosystems are under increasing pressures of pollution and eutrophication (Vidal et al. 1999, Paerl et al. 2006). The latter, a problem first limited to enclosed or semi enclosed water bodies, is now being observed in most coastal areas (Valiela et al. 2000, Cloern 2001, Druon et al. 2004, Gilbert et al. 2010). Determining the origin of the dissolved nitrogen in estuarine environments can be an effective means of evaluating nutrient management policies, and may ultimately lead to more successful environmental regulation of anthropogenic nitrogen (Ahad et al. 2006).

The adverse effects of anthropogenic nitrogen inputs have led to the development of suitable indicators to assess water quality of aquatic ecosystems, both for management or biological issues. Direct quantification of dissolved inorganic nitrogen in water has been frequently used (e.g. Hickel et al. 1993, Rabalais et al. 1996, Paerl et al. 2006). However, nutrient concentrations in the water column alone seem not to be adequate to quantify anthropogenic loads as they are highly variable in time because of rapid consumption by primary producers (Fry et al. 2003). Moreover, changes in nitrogen concentrations may not only be due to anthropogenic inputs but also to natural processes, as coastal upwelling (e.g. Arístegui et al. 2006).

As an alternative to nutrient measurement, the ratio of nitrogen stable isotopes ($\delta^{15}N$) in macroalgae has been increasingly used to quantify the importance of different nitrogen sources for primary producers (McClelland et al. 1997, McClelland and Valiela 1998a, Tucker et al. 1999, Riera et al. 2000, Gartner et al. 2002, Savage and Elmgren 2004, Constanzo et al. 2005, Lapointe and Bedford 2007, Piñón-Gimate et al. 2009). Nitrogen has two stable isotopes, and its proportion might vary according to the different metabolic routes that a molecule follows, as light isotopes (^{14}N) are mobilized faster by some processes than the heavy ones (isotopic fractionation). For some biological reactions, the reactants are progressively enriched in heavy isotopes while the products are relatively depleted at a rate characteristic of each reaction (Mariotti et al. 1981). Anthropogenic nitrogen sources, like sewage, manure, terrestrial runoff, fish farm waste and groundwater, are often more enriched in ^{15}N than seawater (Heaton 1986, Jordan et al. 1997, Voβ and Struck 1997, McClelland

and Valiela 1998a, Vizzini and Mazzola 2004) because of isotopic fractionation during nitrification and volatilization in the case of NH_4^+, or denitrification in the case of NO_3^- (Montoya 2008). In contrast, nitrogen pools from most agricultural facilities are characterized by depleted isotopic values, as they are synthesized from atmospheric N_2 (Heaton 1986). Furthermore, $\delta^{15}N$ in macroalgae can also be used to detect the intensity and variability of the anthropogenic nitrogen loading (Cole et al. 2004, Savage and Elmgren 2004, Costanzo et al. 2005) often related to the degree of urbanization in the watershed (McClelland et al. 1997, McClelland and Valiela 1998a, Cole et al. 2004, 2005).

Besides nutrients from anthropogenic origin, different natural processes also affect inorganic nitrogen concentrations and in consequence macroalgal isotopic values. For instance, algae from mangrove habitats that were exposed to nitrogen derived from N_2 fixation were depleted in ^{15}N while those in habitats with frequent coastal upwelling were relatively enriched (Lamb et al. 2012). In addition, $\delta^{15}N$ in estuarine waters vary as a consequence of freshwater inputs and local biogeochemical processes (Ahad et al. 2006). Because different combinations of sources may produce similar $\delta^{15}N$ values, additional information on factors affecting local nitrogen dynamics is required to obtain unequivocal evidence that significant amounts of anthropogenic nitrogen are affecting the coastal zone.

The regions of Galicia and Asturias (NW Spain, Fig. 2.1) are characterized by the presence of estuaries and rias sustaining high levels of biological production due to seasonal upwelling fertilization (Arístegui et al. 2006). Each of these rias has also an independent river basin, but the nutrient inputs from these rivers are lower than those from the upwelling (Bode et al. 2011b). The upwelling has a larger impact in the production of western and southern rias (Galicia) because the initial nutrient inputs are amplified by remineralization of organic matter in the shelf and subsequent import with estuarine circulation (Álvarez-Salgado et al. 1997). In contrast, upwelling in the northern coast (Asturias) is generally weaker than in the western coast and limited to the vicinity of major capes (Botas et al. 1990). Upwelling nutrients support a larger fraction of primary production in Galicia than in Asturias (Álvarez-Salgado et al. 2002, Bode et al. 2011a). In consequence, geographic variability in the nitrogen sources, and correspondingly in their isotopic signature, can be expected in NW Spain. Besides, most of the human population concentrates in the coastal zone, which showed large urbanization development

in recent years (Viña 2008). Previous studies of macroalgal $\delta^{15}N$ in this region reported high enrichment near large urban areas and inside the rias, suggesting the influence of nitrogen from wastewater (Bode et al. 2006, Bode et al. 2011b, Viana et al. 2011, Carballeira et al. 2013).

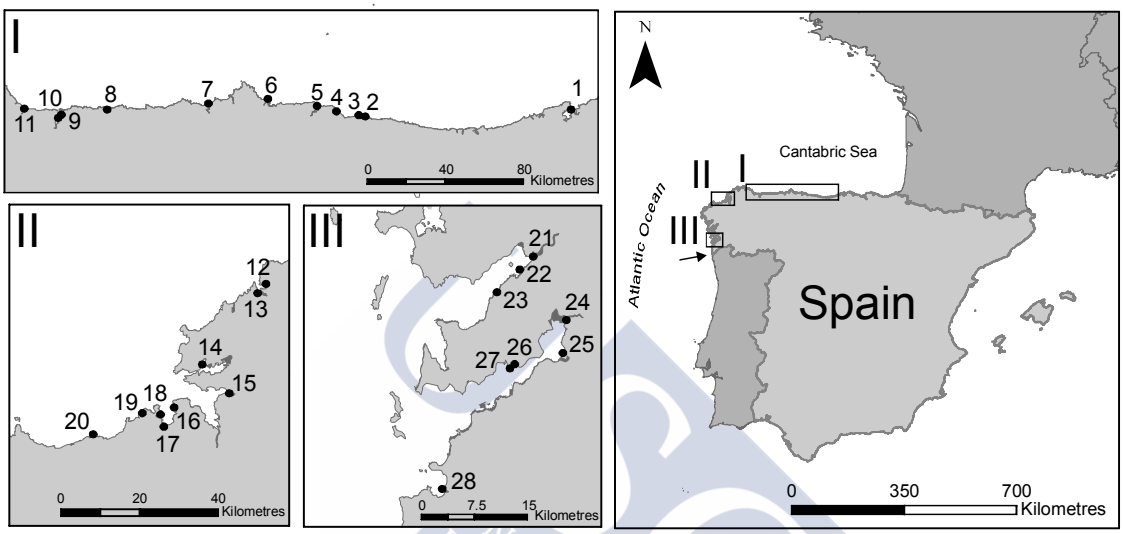

Figure 2.1. Location of sampling sites along NW Spain. Three environment types representing coastal sites in large rias (I), sites in or near middle rias (II) and mostly open sea sites at the northern coast (III) were considered. The arrow indicates the River Miño discharge point used as the southernmost reference point to compute intersite distances in this study.

In this study the variability in the isotopic composition of two intertidal macroalgae in relation to concurrent measurements of dissolved inorganic nitrogen concentrations and isotopic composition in the NW coast of Spain was analyzed to determine the relative importance of anthropogenic versus natural nitrogen sources. The effect of the coastal upwelling, as the main natural source of nitrogen, was represented by the geographical distribution of sampling sites along the coast, while the main anthropogenic input was represented by the size of the human population in the watershed as a proxy for wastewater production.

Material and methods

Sampling

Samples were collected in the intertidal along the coast of NW Spain at sites representative of environments with variable influence of the upwelling and in a large range of urban influence (Fig. 2.1). As upwelling in the northern coast is generally weaker than in the western coast (Botas et al. 1990), an arbitrary reference point located at the sea discharge point of the River Miño (Fig. 2.1) was used to compute the distance along the coast between each sampling site and this reference point. This distance was intended to indicate the lower input of new nitrogen by the upwelling in the northern coast (Mar Cantábrico, zone I in Fig. 2.1) compared to those in the western coast (Galicia). In the latter, two zones were considered to investigate potential differences between Rías Baixas (zone III) and other rias (zone II). Sampling sites covered a large range of urban population influence in the watershed (from ~240 to ~246,000 inhabitants) according to Spanish Official Population Census (http://www.ine.es/inebase). Sampling surveys were carried out mostly during spring and summer 2010 and 2011, but some samples from 2006 were added to complete the range of geographic or urban population values (Table 2.1).

Two species of Phaeophyceae (brown algae) were selected: *Ascophyllum nodosum* and *Fucus vesiculosus*. The species were present at 12 and at 26 sites respectively, and they were cohabiting at 11 sites. Three individuals of each macrophyte species fixed to the substrate were collected from the meso-littoral zone when emerged. Apical parts of the specimens (1 cm) were used for analysis of the stable nitrogen composition. Samples were rinsed with Milli Q water to remove sediments and other material and frozen (-20 °C) before processing. Samples were defrosted and dried (50 °C) until constant weight, before grinding into a homogeneous powder.

Samples of surface water were collected concurrently with macroalgae. Salinity was measured *in situ* with a portable conductivity meter (YSI Model 30). Water samples were poisoned with $HgCl_2$ (0.05% final concentration) to prevent microbial alteration and stored in tightly caped Pyrex flasks.

Table 2.1. Mean (±se) values of total nitrate (NO_3^-+NO_2^-) and ammonium (NH_4^+) concentrations and $\delta^{15}N$ in water and macrophyte samples at the sampling sites. Salinity (S) and the number of inhabitants in the watershed (population) are also indicated. Code is the number of each site in Fig. 2.1.

							Concentration (µM)		$\delta^{15}N$			
Code	Site	Latitude	Longitude	Date	Population	S	NO_3^- + NO_2^-	NH_4^+	NO_3^- + NO_2^-	NH_4^+	*A. nodosum*	*F. vesiculosus*
1	El Sardinero	43.48145	-3.78715	11/05/2011	141,269	34.3	2.15±0.02	-	-	-	-	5.8±0.3
2	Toró	43.41743	-4.74270	12/05/2011	276	34.9	3.63±0.51	-	-	-	-	4.5±0.1
3	El Sablón	43.42247	-4.75226	12/05/2011	5,358	33.8	3.63±0.04	-	-	-	-	5.5±0.1
4	La Griega	43.50288	-5.26320	06/08/2010	3,878	33.9	4.33±0.60	≥10	5.1±0.3	-	-	4.5±0.2
5	El Puntal	43.52605	-5.38812	06/08/2010	239	32.7	1.80±0.25	≥10	4.0±0.2	-	6.1±0.3	-
6	Xivares	43.56827	-5.71207	05/08/2010	2,675	34.2	5.36±0.75	≥10	4.8±0.3	-	-	-
7	S. Juan de la Arena	43.55705	-6.07709	16/04/2010	1,970	5.3	19.68±2.74	4.03±0.55	4.5±0.2	-	-	10.5±0.1
8	Navia	43.55214	-6.72481	16/04/2010	8,906	1.8	22.37±3.12	2.28±0.31	3.7±0.2	-	-	6.2±0.2
9	Figueras	43.53794	-7.02360	16/04/2010	3,845	29.8	14.80±2.06	2.82±0.38	18.0±1.0	-	5.1±0.2	6.9±0.1
10	Ribadeo	43.53539	-7.03596	31/07/2010	9,983	29.3	9.50±1.32	≥10	6.2±0.3	-	-	6.8±0.6
11	Foz	43.56468	-7.24599	31/07/2010	13,214	27.2	14.40±2.01	2.75±0.37	3.7±0.2	-	5.7±0.3	6.4±0.1
12	Cedeira	43.66007	-8.05606	16/08/2010	7,465	31.7	4.28±0.60	≥10	6.5±0.3	-	-	13.8±0.6
13	Vilarrube	43.64518	-8.08386	16/08/2010	363	28.6	5.83±0.81	3.67±0.50	2.5±0.1	-	7.8±0.2	8.2±0.0
14	A Graña	43.47893	-8.26019	25/07/2010	74,273	34.5	4.14±0.58	≥10	2.9±0.2	0.3±0.1	7.7±0.1	7.2±0.4
15	Cabanas	43.41146	-8.17255	17/08/2010	11,793	29.6	7.20±1.00	≥10	4.9±0.3	-	6.2±0.3	6.9±0.2
16	Mera	43.38247	-8.34397	18/04/2011	32,947	32.8	14.74±2.05	3.51±0.48	4.7±0.3	-	-	8.2±0.0
17	O Burgo	43.32770	-8.37034	18/04/2011	83,691	26.5	39.38±5.49	4.72±0.64	3.3±0.2	-	8.9±0.1	9.5±0.0
18	A Coruña	43.36916	-8.38836	16/02/2006	243,349	-	1.92±0.27	4.85±0.66	-	-	-	8.0±0.2
19	Bens	43.36926	-8.45777	26/07/2010	246,056	35.6	5.93±0.83	≥10	4.8±0.3	0.8±0.1	-	4.8±0.2
20	Caión	43.31825	-8.60719	15/02/2006	661	-	3.10±0.43	7.47±1.02	-	-	-	4.7±0.0
21	Pontevedra	42.42799	-8.65340	24/07/2010	81,756	26.4	11.80±1.64	≥10	4.0±0.2	-1.6±0.1	-	8.9±0.2
22	Placeres	42.40659	-8.68541	10/08/2010	16,996	35.3	3.29±0.46	4.25±0.58	4.7±0.3	-	7.2±0.2	6.5±0.2
23	Aguete	42.37571	-8.72958	10/08/2010	1,075	35.6	1.40±0.20	≥10	4.8±0.3	-	-	6.4±0.1
24	Soutomaior	42.34022	-8.61412	24/07/2010	6,867	26.5	5.22±0.73	≥10	5.7±0.3	2.4±0.1	-1.6± 0.4	1.6±1.3
25	Cesantes	42.29945	-8.61677	24/07/2010	30,001	35.5	5.61±0.78	≥10	6.5±0.3	2.6±0.2	9.9±0.3	8.7±0.2
26	O Latón	42.27885	-8.70626	28/08/2010	19,014	2.4	7.10±0.99	≥10	19.6±1.0	-	-	9.5±0.5
27	Meira	42.27654	-8.71091	18/02/2006	18,415	-	2.27±0.32	4.12±0.56	-	-	10.1±0.1	8.0±0.2
28	Ramallosa	42.12180	-8.81998	24/07/2010	18,021	32.5	10.85±1.51	≥10	5.3±0.3	1.9±0.1	10.1±0.9	10.2±0.1

Chemical analysis

Nitrate, nitrite and ammonium were determined in the laboratory using segmented flow analysis (Braun-Luebbe AAII) following the procedures of Grasshoff et al. (1983). Sensitivity was 0.05, 0.01 and 0.04 μM for nitrate, nitrite and ammonium, respectively. Precision (se of 3 replicates) was better than 14% of the mean value for any of the nitrogen species. Ammonium values >10 μM were excluded from further analysis because of suspect contamination of samples during processing, as values reported for coastal waters in the study region do not exceed 10 μM (e.g. Bode et al. 2011b).

The isotopic composition of total nitrate (NO_3^-+NO_2^-) was determined by previous conversion into ammonium and later recovery of ammonium on a solid phase. The procedure is an adaptation of the diffusion method (Sigman et al. 1997) involving the incubation of samples in two steps. In this case the resulting ammonium was collected on a small disk of glass-fiber filter placed in the gas headspace of the diffusion flask (Slawyk and Raimbault 1995). First, aliquots of the samples were incubated (50 °C, 1 week) in the same collecting flask without cap to reduce the volume and concentrate nitrate. Ashed MgO was added to raise pH above 9.7 to remove ammonia by volatilization. In the second step (50 °C, 2 weeks), ashed Devarda´s alloy was added to the reduced volume sample to convert nitrate and nitrite into ammonium. The high pH (>11) of the mixture ensured also the conversion of ammonium into ammonia gas that was collected on a sterilized glass-fiber disk (Whatman GF/F), acidified with 0.5 ml of 0.25N H_2SO_4 and hooked on a needle fixed to the inner side of the flask cap. Care was taken to ensure that the filter disk did not contact the liquid sample. This extraction procedure does not allow separation between NO_3^- and NO_2^- therefore the values reported are the combined isotopic signatures of total nitrate (Ahad et al. 2006). After the second incubation step the disk filters were dried and prepared for isotopic analysis. The stable isotope composition of ammonium was determined in another aliquot of the water samples by an adaptation of the diffusion method (Holmes et al. 1998). This method involves gas-phase diffusion as described for the second step of the total nitrate extraction. In all cases corrections for isotopic fractionation during the whole incubation and diffusion steps were made (Holmes et al. 1998). The measured values of natural abundance of dissolved inorganic nitrogen were retained for further analysis when the ammonium recovery after the diffusion procedure exceeded 45% and isotopic fractionation of internal standards was within 1‰ of values estimated from the empirical equation in Holmes et al. (1998).

Stable isotopes

The natural abundance of stable nitrogen isotopes was determined in macroalgae and water samples (total nitrate and ammonium). For macroalgae, 2.5 mg of dry sample was analyzed to ensure a minimum of 10 µg of N. For water samples, 1 ml of 4 mM-N $(NH_4)_2SO_4$ was added to each sample during the diffusion phase to ensure the detection limit was achieved. Samples were placed in tin capsules and introduced into an isotope-ratio mass spectrometer (Thermo Finnigan Mat Delta Plus) via an element analyzer (Carlo Erba CHNSO 1108). Isotopic results are expressed in delta notation:

$$\delta^{15}N = [(^{15}N_{sample}:^{14}N_{sample}/^{15}N_{std}:^{14}N_{std})-1] \times 1000$$

where the standard (std) for $\delta^{15}N$ is atmospheric N_2. Precision (se of 5 replicates) was better than 0.05‰ for either IAEA-N-2, IAEA-N-1 or IAEA-NO-3 standards. The coefficient of variation of triplicate sample aliquots was always <2%.

Statistical procedures

Relationships between variables were first analyzed using non parametric correlation (Spearman ρ). Further analyses were made using linear regression after excluding outliers exceeding 1.5 times the interquartile range. In the case of salinity vs. dissolved nitrogen concentrations and macroalgal $\delta^{15}N$ vs. geographical distance, product-moment regression was used because either the error in estimating the salinity was much lower than the error for dissolved nitrogen or because the resulting slope was further employed to account for systematic variability in $\delta^{15}N$ with geographical distance (Sokal and Rohlf 1981). In the case of the comparison of $\delta^{15}N$ between the two macroalgal species standard major axis was used because both variables were measured with the same type of error (Sokal and Rohlf 1981). In this latter case, the obtained regression parameters were compared with the line of slope 1 and zero intercept by a *t*-test (Warton and Ormerod 2007).

The relative contribution of geographical distance and population size to $\delta^{15}N$ was estimated as the sums of squares (Type I) obtained with an ANOVA design including two population size classes (larger and smaller than $15x10^3$ inhabitants, respectively) with distance as covariable. Differences between sampling zones or classes of population size were further analyzed by non parametric Kruskal-Wallis (K-W) test (Sokal and Rohlf 1981).

Results

Dissolved inorganic nitrogen

Total nitrate concentration in the samples ranged from 1.40 to 39.38 µM, while ammonium (excluding >10 µM values) ranged from 2.28 to 7.47 µM (Table 2.1). Total nitrate was negatively correlated with salinity in most samples (Spearman ρ =-0.682, P<0.001, n=24) except at O Burgo, where nitrate reached ca. 40 µM (Fig. 2.2). In contrast, ammonium was not correlated with salinity (P>0.05). These relationships with salinity suggest large potential contributions of nitrate from freshwater in most of the studied area but variable inputs of ammonium unrelated to freshwater discharges.

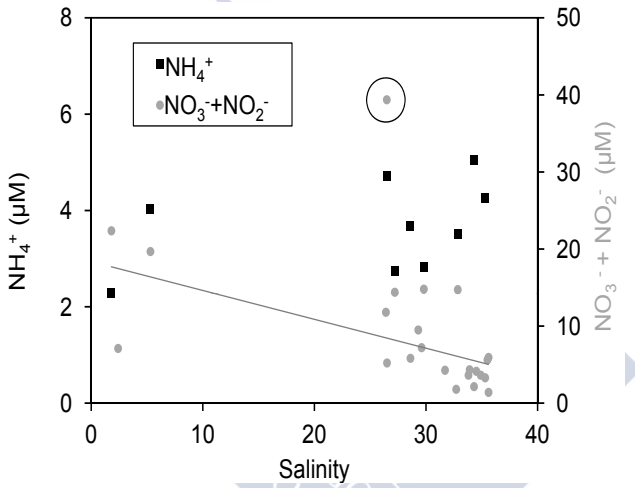

Figure 2.2. Linear relationships between ammonium (NH_4^+, black squares) or total nitrate (NO_3^-+NO_2^-, gray circles) and salinity in water from the sampling sites. The point encircled was an outlier (>1.5 times the interquartile range) not used in the estimation of the regression line (Spearman ρ=-0.682, P<0.001).

Because of rapid contamination with ambient ammonia during the analytical preparation steps stable isotope composition of dissolved nitrogen was determined with confidence in a subset of samples only (Table 2.1). Total nitrate $\delta^{15}N$ varied between 2.5 and 19.6‰ while $\delta^{15}N$ ammonium ranged from -1.6 to 2.6‰ (Table 2.1). When measured concurrently $\delta^{15}N$ of ammonium and $\delta^{15}N$ of total nitrate were correlated (Spearman ρ=0.943, P<0.01, n=6). The highest nitrate value corresponded to the sample from O Latón (Code 26), collected at the discharge outlet of a Water Treatment Plant, but a large value was also observed in Figueras (Code 9), in this case not obviously related to residual water discharges. Values of nitrate $\delta^{15}N$ for marine waters (salinity >35) were near 5‰.

$\delta^{15}N$ in macroalgae

Stable isotope composition of *F. vesiculosus* and *A. nodosum* was significantly correlated (Spearman ρ=0.806, P<0.01, n=10). The resulting regression line did not differ from a line with slope 1 and intercept 0 (P<0.05) indicating that the isotopic composition of these species was equivalent for a given site (Fig. 2.3). In contrast, macroalgal $\delta^{15}N$ was not correlated with either dissolved inorganic nitrogen concentrations, salinity or isotopic composition (Fig. 2.4).

Geographic variability in $\delta^{15}N$

Macroalgal $\delta^{15}N$ varied according to the geographical location of samples (Fig. 2.5). Both species showed a linear decrease in $\delta^{15}N$ with the distance from the reference point in the River Miño (Fig. 2.5a). The slope of the regression lines indicated a change of $\delta^{15}N$ of 0.3 and 0.4‰ per 100 km of coastline for *F. vesiculosus* and *A. nodosum* respectively (Table 2.2). In contrast a significant relationship was not found between dissolved nitrogen concentrations or $\delta^{15}N$ of total nitrate with distance, as exemplified by total nitrate concentration (Fig. 2.5b). No significant differences resulted either when considering the sampling zones (I, II and III) in a K-W test (P>0.05).

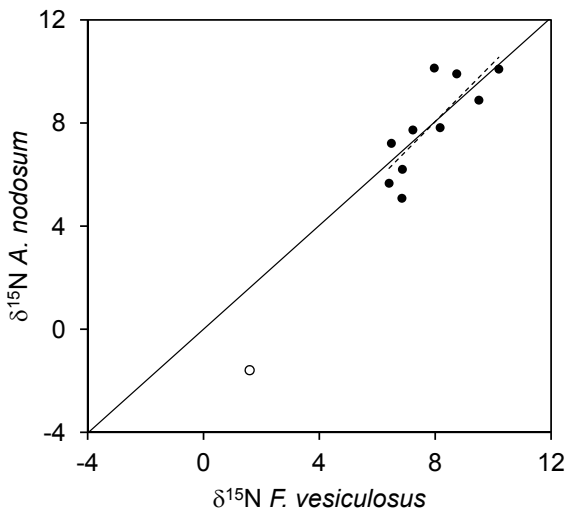

Figure 2.3. Relationship between stable isotope composition of *Ascophyllum nodosum* and *Fucus vesiculosus* sampled at the same locations. The regression line computed without the outlier (open circle, >1.5 times the interquartile range) is significant and with zero intercept (Spearman ρ=0.806, P<0.01) while the slope is non-significantly different from 1.

Samples of *F. vesiculosus* collected inside the rias and estuaries (as shown in Fig. 2.1) had higher $\delta^{15}N$ values than samples collected in open coastal sites (K-W test, $P<0.01$). Mean (±se) values for rias and coastal sites, after correction for the geographic variability using the slope in Table 2.2, were 9.1±1.1‰ (n=17) and 7.6±1.1‰ (n=7), respectively.

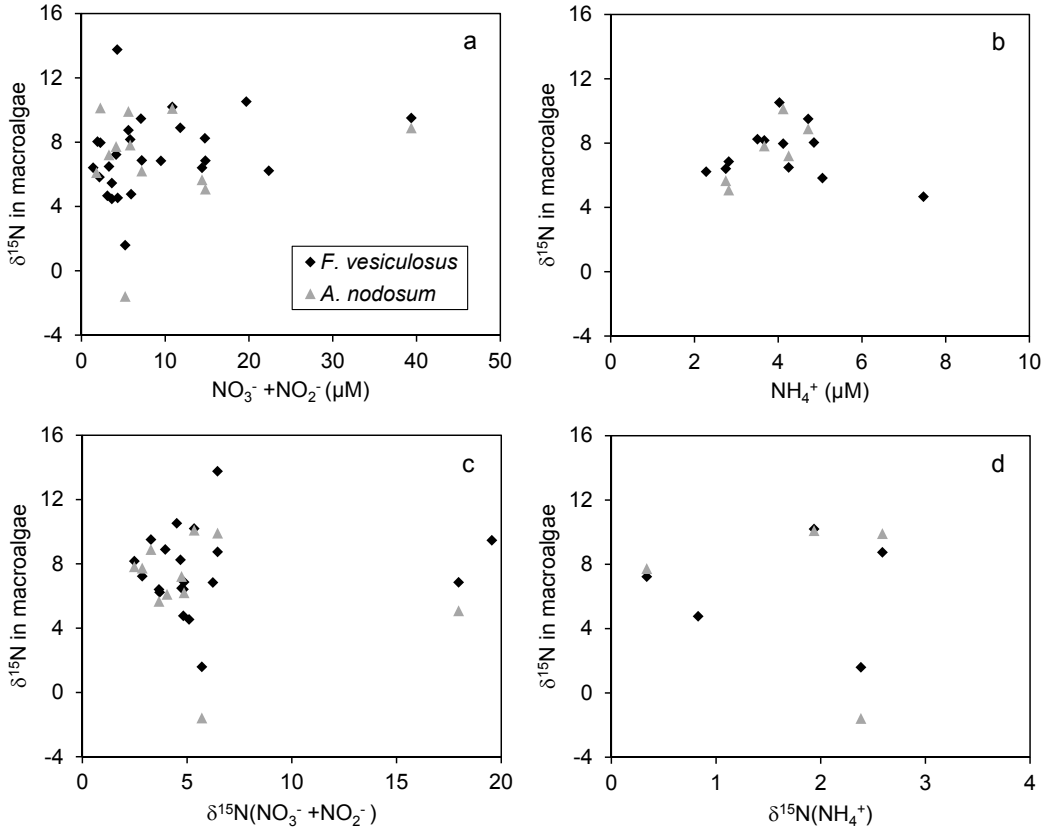

Figure 2.4. Biplots of macroalgal $\delta^{15}N$ and concentrations of total nitrate (a) and ammonium (b) or $\delta^{15}N$ in total nitrate (c) and ammonium (d). None of the relationships is significant (Spearman ρ, $P>0.05$).

Variability of $\delta^{15}N$ with human population

The geographic variability accounted for more than half of total variance in $\delta^{15}N$ for both species (Fig. 2.6). However, the size of the human population in the watershed was also an important factor for $\delta^{15}N$, particularly for *A. nodosum*. The isotopic values of both macroalgae, after removal of the geographic trend using the equations in Table 2.2, increased non-linearly with the size of the human population in the watershed (Fig. 2.7).

Variability in $\delta^{15}N$ was largest at small population sizes (<50x10^3 inhabitants) with clear outliers with unusually large or small values. At the three sites influenced by large populations (>100x10^3 inhabitants) $\delta^{15}N$ values in *F. vesiculosus* (as *A. nodosum* was not found at these sites) did not follow the increase observed at lower populations. In turn, the distribution of the human population has no relationship with the geographical gradient found for macroalgal $\delta^{15}N$ (no significant correlation between population size and distance). In any case, and excluding the outliers, both species showed significantly higher $\delta^{15}N$ values at population sizes larger than 15x10^3 inhabitants (Fig. 2.8, K-W test, P<0.05).

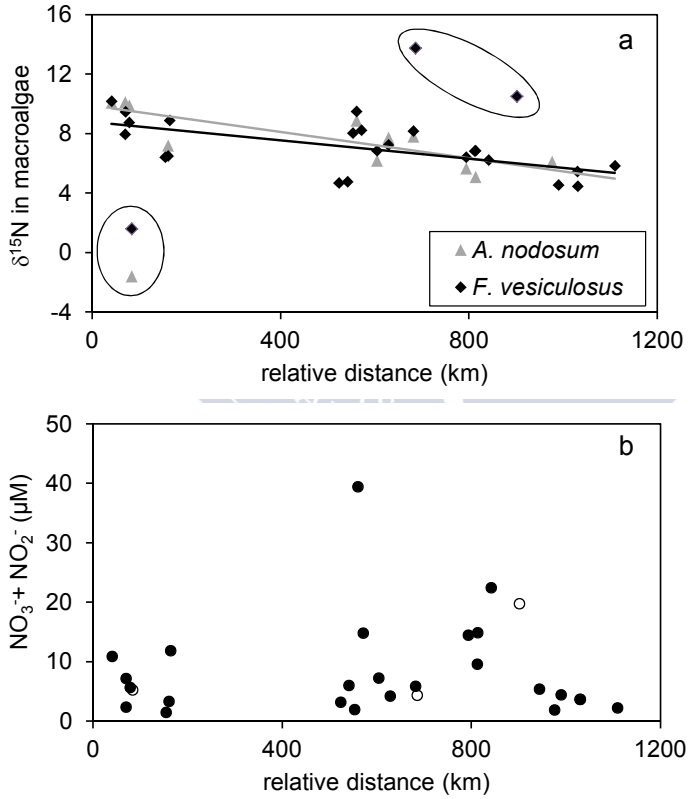

Figure 2.5. Variability of $\delta^{15}N$ in macroalgae (a) or total nitrate (b) with the relative distance of sampling locations to the River Miño discharge point (see Fig. 2.1). The regression lines for *Ascophyllum nodosum* (Spearman ρ=-0.855, P<0.01) and *Fucus vesiculosus* (Spearman ρ=-0.590, P<0.01) are indicated. Outliers of $\delta^{15}N$ (>1.5 times the interquartile range and not used in the estimation of regression lines) are enclosed in circles (a) while the corresponding inorganic nitrogen concentrations are shown as open dots (b).

Table 2.2. Linear regression parameters ($\delta^{15}N$ = a + b distance) of the variation of $\delta^{15}N$ in *Fucus vesiculosus* and *Ascophyllum nodosum* with the distance in km to the River Miño. P: significance, n: number of data points, se: standard error. The outliers in Fig. 2.5 were excluded from the estimation.

species	a±se	b±se	r	P	n
F. vesiculosus	8.774±0.530	-0.003±0.001	0.639	0.001	23
A. nodosum	9.889±0.610	-0.004±0.001	0.819	0.002	11

Discussion

Natural variability of nitrogen sources

Differences in both concentration and $\delta^{15}N$ values of nitrate were expected in the NW Spanish coast because of the varying influence of the upwelling, as nitrate from the Eastern North Atlantic Central waters is the main natural source of nitrogen for primary production in shelf waters of this area (Botas et al. 1990, Casas et al. 1997, Álvarez-Salgado et al. 2002). Instead, our results indicated no significant spatial variability pattern of nitrate concentrations or $\delta^{15}N$. Nitrate was the main form of dissolved inorganic nitrogen and its highest concentrations were found in estuarine waters, suggesting a significant input from freshwater. However, given the low flow of rivers in

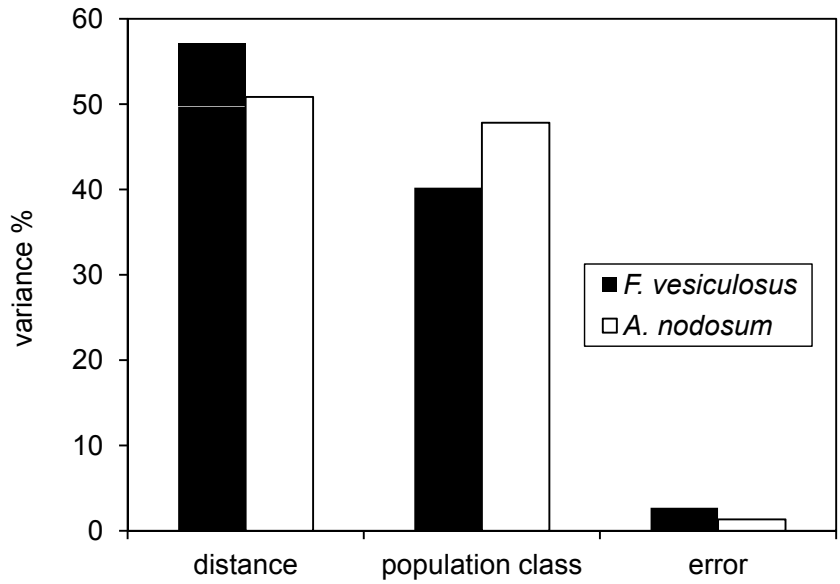

Figure 2.6. Contribution of distance to the reference point (as covariable) and human population (as fixed factor with two levels: larger and smaller than $15x10^3$ inhabitants, respectively) to the variance of $\delta^{15}N$ in *Fucus vesiculosus* and *Ascophyllum nodosum*. The error term includes the remaining variability not accounted for by all other components. The outliers in Fig. 2.5 were not included in the analysis (ANOVA, $P<0.05$ for all components).

this region (Rio Barja and Rodríguez Lestegás 1996) the influence of riverine nitrate can be considered only of local importance, as reported in other studies (Gago et al. 2005, Bode et al. 2011b). This is supported by our $\delta^{15}N$ measurements in nitrate, the first reported for this region, with values close to 5‰ in most cases and particularly in seawater. These values agree with the range reported for subsurface nitrate in the N Atlantic (Liu and Kaplan 1989), while the largest values (>10‰) suggest local influence of nitrate from nitrification of ammonium (Mariotti et al. 1981).

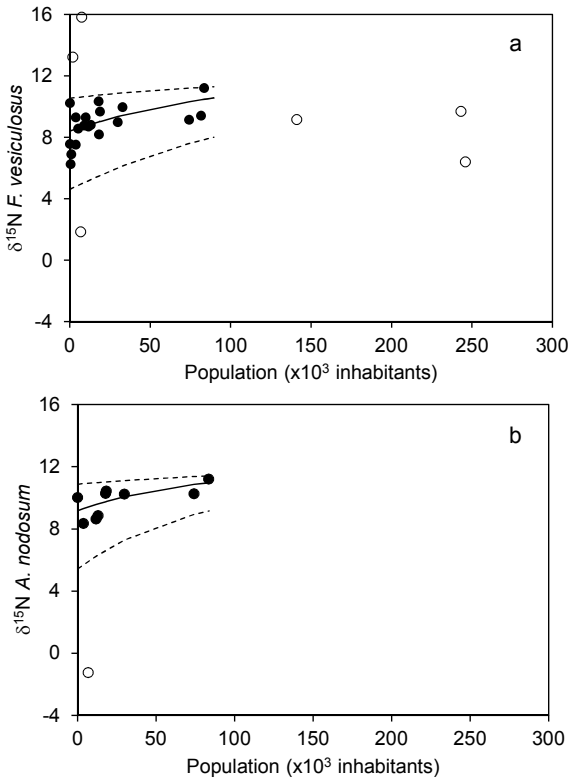

Figure 2.7. Variability of $\delta^{15}N$ in *Fucus vesiculosus* (a) and *Ascophyllum nodosum* (b) with the size of the human population in the watershed. The curves are polynomial (a) or lineal (b) fits and 95% confidence limits only intended for descriptive purposes. Isotopic values were corrected for the geographic variability using the equations in Table 2.2. Open symbols indicate outliers (>1.5 times the interquartile range) not used to fit the curves.

Systematic observations of coastal waters revealed the importance of local, short-term upwelling for nutrient inputs in the study area (Álvarez-Salgado et al. 1997, Casas et al. 1997, Nogueira et al. 1998). Because of this nutrient variability, instantaneous nitrogen concentrations and isotopic composition of water samples are not directly reflected in macroalgae collected in the field, in contrast to the findings in laboratory

experiments allowing for isotopic equilibration between water nitrogen and algal tissues (Cohen and Fong 2005). Temporal variability in the isotopic composition of inorganic nitrogen is expected to be high, as reported for two northeastern English estuaries (Ahad et al. 2006) and related to changes in either nitrogen sources or in the biogeochemical processing of nitrogen. Such variability and the rapid turnover of surface waters in the region would prevent isotopic equilibration and therefore a close correspondence between the isotopic composition of single water samples and those of macroalgal tissues that integrate isotopic composition over time would not be expected. Both *A. nodosum* and *F. vesiculosus* are long-lived and perennial macroalgae. Individual fronds can become up to 15 (*A. nodosum*) and 3 years old (*F. vesiculosus*) before breakage (Niell 1979, Keser and Larson 1984a). Both species have apical growth (Moss 1965, Strömgren and Nielsen 1986), so the sampled apical tips integrate nutrient concentration and isotopic values from the water nutrients during their growing period. This period can be calculated from their growth rates. *F. vesiculosus* growth shows pronounced latitudinal differences (Mathieson et al. 1976), but at latitudes similar to the study area it ranges between 0.6 and 2.8 cm mo^{-1} (Knight and Parke 1950, Fuentes 1986). *A. nodosum* growth rates average 10 cm yr^{-1} (Niell 1979) thus implying that the observed $\delta^{15}N$ values are the result of the integration of nitrogen inputs during one month period approximately. In our study macroalgae showed a general ^{15}N depletion along the coast (Fig. 2.5), following the higher prevalence of upwelling in the southern areas compared to those in the northern coast. Therefore, the integration at monthly time scales reflects nitrogen sources more appropriately than water samples. Similar isotopic gradients were observed in intertidal species in other upwelling regions (Hill and McQuaid 2008).

Anthropogenic nitrogen inputs and macroalgal $\delta^{15}N$

Notwithstanding the frequent use of macroalgal $\delta^{15}N$ as a tracer for anthropogenic nitrogen in coastal ecosystems in the last decades, only few studies showed experimental evidence of isotopic enrichment in algal tissues after exposure to enriched dissolved nitrogen (Gartner et al. 2002, Naldi and Wheeler 2002, Cohen and Fong 2005). Instead, many studies report the progressive change in $\delta^{15}N$ of macroalgae with distance of a clearly identified wastewater discharge point (e.g. Riera et al. 2000, Gartner et al. 2002, Savage and Elmgren 2004, Constanzo et al. 2005, Carballeira et al. 2013). When anthropogenic nitrogen was provided by diffuse or pulse inputs (e.g. from groundwater) over a relative large area, other studies showed a direct relationship between the size of the anthropogenic load (estimated from computation in the watershed) and

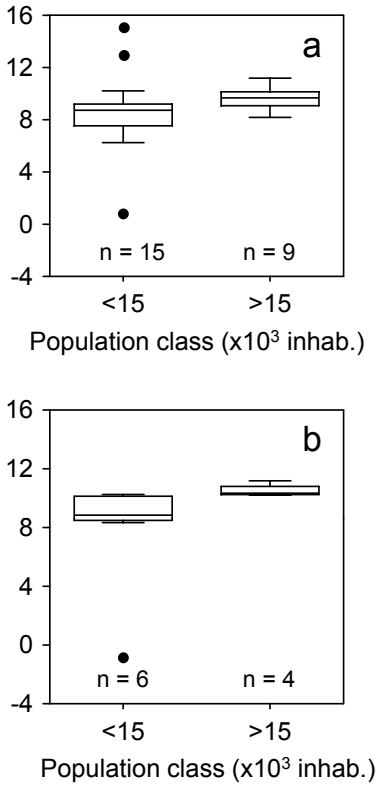

Figure 2.8. Box and whisker plots of $\delta^{15}N$ in *Fucus vesiculosus* (a) and *Ascophyllum nodosum* (b) grouped according to the size of the human population in the watershed. The differences between classes are significant for both species (Kruskal-Wallis test, $P<0.05$).

macroalgal $\delta^{15}N$ (McClelland et al. 1997, McClelland and Valiela 1998a, Cole et al. 2004, 2005), as the degree of urbanization affects $\delta^{15}N$ of groundwater nitrate (McClelland and Valiela 1998a, Cole et al. 2006). In the latter case, the use of direct measurements of concentration or $\delta^{15}N$ in the water would not reveal clear anthropogenic influence because of the relatively low loading rates. The lack of direct correspondence between water concentrations and isotopic composition and macroalgal $\delta^{15}N$ in our study suggests that the inputs of isotopically enriched nitrogen are from diffuse sources. While the influence of other natural sources of nitrogen, as runoff or precipitation with different isotopic signatures cannot be discarded, in the absence of specific data on concentrations and isotopic composition of dissolved nitrogen in freshwater of the study region, the relatively high salinity found in most samples (Table 2.1) would support a minor role of freshwater nitrogen in coastal food webs.

Dissolved nitrogen from urban wastewater generally shows $\delta^{15}N$ values exceeding 10‰ (e.g. Tucker et al. 1999, Gartner et al. 2002, Savage and Elmgren 2004). Similarly high values were reported for manure and other organic fertilizers used in agriculture (Kendall 1998). In our study the sampled nitrate from a water treatment facility (19.6‰) can be considered representative of wastewater nitrogen, and it was considerably enriched when compared to macroalgal samples (Table 2.1). Therefore it can be interpreted that the sampled macroalgae reflect the assimilation of variable fractions of nitrogen from anthropogenic and marine sources. The amount of nitrogen derived from each source could be estimated using a mixing model to compare the measured macroalgal $\delta^{15}N$ with that of marine or wastewater nitrogen, as done in other studies (e.g. Gartner et al. 2002, Savage and Elmgren 2004, Bode et al. 2011b). However, we showed that there was a significant geographic trend of macroalgal $\delta^{15}N$ (but not in other variables) that must be taken into account when performing further estimations in this region (Table 2.2).

The influence of anthropogenic sources is evidenced by the higher $\delta^{15}N$ in macroalgae from rias compared to those in open waters, when the effect of geographical variability is identified. This result agrees with the increasing nitrogen load from anthropogenic sources found in other estuaries (McClelland et al. 1997, McClelland and Valiela 1998a, Cole et al. 2004) and confirms the results from previous studies in the Galician rias (Bode et al. 2006, 2011b). As most of the population concentrates near the rias (Viña 2008) it is not surprising that there was a relationship between the number of inhabitants and macroalgal $\delta^{15}N$. This relationship, however, is not a simple function of the size of the population, and thus on the potential load of wastewater nitrogen, as found in other studies (McClelland et al. 1997) and a large range of $\delta^{15}N$ values was observed below 15,000 inhabitants. Highly ^{15}N enriched isotope values close to small populations (e.g. S. Juan de la Arena, Cedeira, Ramallosa; Table 2.1) might be due to inefficient or lacking treatment of wastewater before disposal, regardless of the population size, as reported in other studies (Savage and Elmgren 2004, Costanzo et al. 2005).

Depleted $\delta^{15}N$ values (e.g. Soutomaior $\delta^{15}N$= -2‰ in *A. nodosum* and +2‰ in *F. vesiculosus*, Table 2.1) may indicate other sources of nitrogen. One possible source would be synthetic fertilizers ($\delta^{15}N$= 1 to 2.6‰, Heaton 1986) but they are much less used in the study area than manure (Nuñez Delgado 2002). Another depleted source would be atmospheric nitrogen, as macroalgae found in oligotrophic

ecosystems supported by diazotrophy (e.g. mangroves) have characteristically low $\delta^{15}N$ because of the assimilation of nitrate remineralized from mangrove litter (Lamb et al. 2012). While there are no reports of high atmospheric nitrogen fixation in the study area, most likely depleted $\delta^{15}N$ may result from high isotopic fractionation during assimilation of a large pool of dissolved nitrogen. Experimental studies have shown that the assimilation of nitrate caused a decrease in algal $\delta^{15}N$ between 0 and 20‰ both in phytoplankton (Waser et al. 1998, Needoba et al. 2004) and macroalgae (e.g. Naldi and Wheeler 2002) with the highest values associated to high nitrogen concentrations. High isotopic fractionation is expected at Soutomaior, located at the innermost zone of the Ría de Vigo, and characterized by high dissolved nitrate concentrations likely resulting from organic matter remineralization in the sediments (Gago et al. 2005). Isotopic fractionation is not generally considered in estimations of source contributions to macroalgal nitrogen (e.g. Gartner et al. 2002, Savage and Elmgren 2004) but it can largely affect the estimates, as illustrated by our measurements at Soutomaior.

Our wide scale survey of macroalgal $\delta^{15}N$ further supports a dominant role of marine nitrogen in coastal ecosystems of NW Spain, as found in previous studies (Bode et al. 2006, 2011b). Large inputs of anthropogenic nitrogen from wastewater appear limited to local scales, likely related to failures in disposal or treatment procedures. As an example, nitrogen waste for fish farms in Galicia has been traced at scales of a few kilometers with $\delta^{15}N$ in macroalgae (Carballeira et al. 2013) while most macroalgae collected far from dumping sites displayed values similar to marine nitrate (Viana et al. 2011). Because of growing urban pressures wastewater treatment in NW Spain is constantly improving with treatment facilities available not only for large cities but also including urban aggregations of 2,000 inhabitants and less (Augas de Galicia, internet). An indirect evidence of this improvement is the correspondence between macroalgal $\delta^{15}N$ and the number of inhabitants in the watershed when the population exceeds 10^5 inhabitants found in our study. In addition, Viana et al. (2011) noted a general decrease of macroalgal $\delta^{15}N$ in the rias between surveys carried out in 1990 and those in 2007, suggesting a general decrease in the impact of wastewater in this region.

Conclusions

Macroalgal $\delta^{15}N$ integrate nitrogen assimilated at time scales of months, thus better reflecting changes in the available nitrogen from different sources than occasional measurements in the water. However, the interpretation of $\delta^{15}N$ values requires a good knowledge of local and regional factors affecting isotopic signatures. Our study showed that large spatial changes can be due to changes in natural sources, such as the influence of upwelling, while the input of anthropogenic nitrogen is not always related to the size of the human population. These factors are not taken into account in most studies using macroalgal $\delta^{15}N$ to estimate anthropogenic nitrogen impacts in coastal ecosystems. Isotopic fractionation and identification of the main nitrogen processes operating at local spatial scales are also key factors for the interpretation of macroalgal $\delta^{15}N$ because, as pointed out for other systems (e.g. Lamb et al. 2012), $\delta^{15}N$ values alone do not provide unequivocal evidence that large amounts of anthropogenic nitrogen are affecting the coastal zone.

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Ecology of Fucus vesiculosus *(Phaeophyceae) at its southern limit of distribution: Growth and production of the early stages of development**

Abstract

Growth and survival of two populations of *Fucus vesiculosus* were studied at the southern limit of distribution of this species at the Eastern Atlantic Coast. Experimentally denudated areas at an estuarine and a semi-exposed site in an upwelling area (NW Spain) were followed for 17 months. Three different cohorts were detected during the sampling period. Differences among the three cohorts in terms of growth, reproduction and survival were detected; these differences may be due to the different time of appearance of the different cohorts or the presence of a coverage of previously implanted individuals when the second and third cohorts were recruited. Although the growth of the cohorts recruiting in autumn was higher than for the latter cohorts; the individual growth was represented in all cases by a logistic function, as the fastest rates of increase in length occurred during the first six months of life and maximum length was attained after the thallus reached the first year. In the same way, production was maximum for the first cohort, recruiting in autumn, even when it had the lowest survival rate, because of the rapid growth of survivors during spring and summer. For both populations, reproduction was continuous through the year but it was maximum during spring and summer. Protection from waves might have favoured higher production and standing stock biomass values at the estuarine site than at the semi-exposed site, while turnover rates of biomass were higher in the latter. Contrary to these expectations, most of the nutrients available for the studied populations were not related to upwelling. Despite the fast initial growth of new recruits, both populations appeared to be very sensitive to denudation.

KEYWORDS: macroalga, demography, growth, reproduction, production, upwelling

* Viana IG, Fernández C, Bode A (in review) Ecology of *Fucus vesiculosus* (Phaeophyceae) at its southern limit of distribution: Growth and production of the early stages of development. Eur J Phycol

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Introduction

The shifting of environmental variables in the course of ongoing global warming is expected to impact the performance and distribution of numerous species in marine coastal systems (Wahl et al. 2011). Recent studies have predicted a potential northward shift of intertidal canopy-forming macroalgae along temperate North-Atlantic rocky shores, especially in the warm-temperate East-Atlantic region from Portugal to Brittany (Jueterbock et al. 2013). As temperature profoundly influences the survival, recruitment, growth and reproduction of seaweeds (Breeman 1988); the study of the response of populations living on the edge could be very important in terms of changes in species' distribution.

Fucoids are the most representative species covering intertidal rocky shores along the European Atlantic coasts from Iceland to Portugal. At their marginal limit of distribution, the NW Iberian Peninsula, these brown seaweeds show a discontinuous distribution, reappearing in isolated patches related with cold water from the spring-summer upwelling (Lüning 1990). Therefore, these marginal populations have been shown to be often smaller and more fragmented than central populations. As these populations are considered to live under suboptimal conditions, little variations in their environment could be critical. An increase of seawater temperature, a decrease in the upwelling intensity and other factors like biological interactions or physiological tolerances (Lamela-Silvarrey et al. 2012, NiCastro et al. 2013, Araújo et al. 2014) can affect the performance of the individuals and increase the risk of disappearance of local populations (Jueterbock et al. 2013).

Fucus vesiculosus Linnaeus is one of the most common fucoid species and it is usually dominant in the mid intertidal rocky shores at both sides of the North Atlantic Ocean. *F. vesiculosus* is found in a wide range of wave exposures, from sheltered to moderately exposed areas (Bárbara et al. 1995) and tolerates a large range of salinities (Kautsky et al. 1992). This species has been widely studied but most investigations were restricted to central populations (Keser and Larson 1984a, Carlson 1991, Chapman 1995, Lehvo et al. 2001, Lamote and Johnson 2008, Wahl et al. 2011). In marginal areas, previous studies of *Fucus* species were mainly focused on community structure and dynamics (Niell 1977a, Fernández and Niell 1982, Bárbara et al. 1995, Lamela-Silvarrey et al. 2012) or on the morphological plasticity of the species (Seoane-Camba 1966, Cairrão et al. 2009, Araújo et al. 2011). However, few studies focusing on growth, production

or recruitment have been made in these environments (Niell 1977a, Fuentes 1986, Lamela-Silvarrey et al. 2012, Araújo et al. 2014).

Recent studies on genetic variability in this species at its southern limit of distribution suggest population´s different responses in their phenology based on the adaptation to changing habitats and stress tolerance (Billard et al. 2010, Zardi et al. 2013, Jueterbock et al. 2014). The objective of the present study is to quantify growth rates, survivorship, reproduction and production of *F. vesiculosus* at an estuarine and at a semi-exposed site at Ría de A Coruña (Galicia, NW Spain). These sites are in an upwelling area at the southern limit of distribution of this species.

Material and Methods

Study sites

The Ría de A Coruña is 6 km long and 3 km wide and can be divided in a large bay and a small estuarine zone (Cabanas et al. 1987). The bay has a large oceanic influence and has a mean depth of 25 m. The estuarine zone (Ría do Burgo) has a mean depth of 10 m and a sharp salinity gradient due to the discharge of the river Mero, with a mean flow of 204 hm^3 yr^{-1}. The eastern margin of the ria and the estuarine zone are heavily populated (ca. 240,000 inhabitants) while the northern and western margins are characterized by mostly rural landscapes. *F. vesiculosus* is well distributed in the rocky intertidal areas of this ria from semi-exposed to wave protected areas (Bárbara et al. 1995).

The study was conducted at two sites representative of the range of habitats of *F. vesiculosus* in the region. Mera (43°22'N, 8°20'W) is a rocky semi-exposed shore near the outer limit of the bay where *F. vesiculosus* is the dominant macroalga from the mid to the lower intertidal, although the population shows a patchy distribution. Individuals in this area typically lack of air bladders and have been described as *F. vesiculosus* var. *evesiculosus* (Bárbara et al. 1995). The site at Ría do Burgo (43°20'N, 8° 22'W) is located at the sheltered part of the ria where a dense *F. vesiculosus* belt is restricted by the presence of a dense population of *Ascophyllum nodosum* in the upper intertidal zone and by the absence of rocky substrata in the lower zone.

The study period lasted 26 months, starting in November 2010 until December 2012. During a 15-month period (November 2010-January 2012). Monthly or

bimonthly visits to both sites were made to record the growth, density, biomass and reproduction of *F. vesiculosus*, along with some water variables. After this first period, bimonthly visits were made just to record the growth of selected individuals.

At each visit, salinity (±0.1, Practical Salinity Scale) and temperature (±0.1 °C) of surface water were measured *in situ* with a portable conductivity meter (YSI Model 30). Samples of surface water were also collected for further determination of dissolved nutrients (NO_3^-+NO_2^-, NH_4^+ and PO_4^{3-}) in the laboratory following Grashoff et al. (1983).

Intensity of upwelling in the study area was represented by an upwelling index (Lavín et al. 1991) that estimates the Ekman transport of surface water in $m^3\ s^{-1}$ by km of coastline computed from geostrophic winds. We used monthly means of the values of the upwelling index data obtained from the Instituto Español de Oceanografía (http://www.indicedeafloramiento.ieo.es) in a grid of 1°•1° centred at 43°N, 11°W. Positive values of this index indicate upwelling of deep waters near the coast while negative values indicate accumulation of shelf surface waters towards the coast (downwelling).

Size distributions, growth and reproduction

At each sampling site, three 50x50 cm experimental quadrats were randomly set up in the *F. vesiculosus* dominant zone. The position of the experimental quadrats relative to the Lowest Astronomic Tide (L.A.T.) was between 1.62 and 2.30 m at Ría do Burgo, and between 1.25 and 1.56 m at Mera. The experimental quadrats were denudated in October 2010. All macroalgae constituting the initial undisturbed population inside the quadrats were removed as close to the ground as possible and the substrate was cleaned with a metal brush to ensure no small individuals or the holdfast of any adult individual could remain attached. The material obtained was transported to the laboratory in plastic bags and used for the description of the initial undisturbed population. Observations of the accompanying flora along the studied period were also recorded. All removed individuals were measured, and fronds from the same holdfast were considered as an individual. The total biomass of *F. vesiculosus* and accompanying flora was determined as wet and dry weight (±1 g).

During a 15-month period (November 2010 to January 2012) all individuals within each experimental quadrat were measured monthly, excepting in February

and December 2011. All individuals inside the quadrats were mapped and measured to the nearest mm from the base of the holdfast to the tip of the longest frond. During summer sampling surveys, when the abundance in some of the experimental areas was high, individuals shorter than 0.5 cm were counted and 90 of these individuals were measured to determine their mean length. All individuals were classified in size classes 5-mm wide, from <0.5 to >30.5 cm. Density estimations were made by combining all the results obtained in the three experimental quadrats. Five months after the beginning of the experiment (March 2011), 10 randomly selected individuals within experimental quadrats of each site, and that had been already mapped and measured during the previous period, were labelled. Their growth in length was monitored every month until January 2012, and every 2-3 months until December 2012.

At each site, monthly frequency distributions of size classes were calculated from the number of individuals in each size class. Individuals that were first detected in each experimental quadrat were considered as new recruits, and each set of new recruits was monitored as a new cohort. Cohorts were assumed to have normal or log-normal frequency distributions and selection of cohort size ranges was made from the comparison of frequency distributions of consecutive dates.

The growth was monitored by monthly changes of the modal length of each cohort fitted to a logistic equation (Niell 1979):

$$L_t = L_{max} / (1 + e^{(a1 + a2\ Age)})$$

where L_t is the modal length at time *t*, L_{max} is the asymptotic length, and a1 and a2 are constants. GraphPad Prism 4 software was used to estimate the fitted curves.

To estimate the age of maturity and reproductive periods of *F. vesiculosus* in the area, the presence of reproductive tips in individuals of the different cohorts within the experimental quadrats was also recorded during the first 15 months of the sampling period (November 2010-January 2012).

Demography and production

The fate of individuals in the quadrats was monitored based on the monthly maps. New recruits were considered when they were first detected and any individual

that disappeared from the experimental quadrats was considered dead. The survivorship (S) of each cohort was estimated from the date when the maximum abundance (maximum recruitment) was detected. Survivorship was calculated as the fraction of individuals remaining from the cohort maximum recruitment and fitted to an exponential decay function with age:

$$S = S_0 \, e^{-(m \, Age)}$$

where S_0 is the density when the cohort was detected (maximum recruitment) and m is the mortality rate.

The dry weight biomass of each individual (w, g) was determined using a length-weight relationship computed from individuals of different lengths (L, cm) and without receptacles sampled at both sites ($L=15.460\ w^{0.407}$, $r^2=0.938$, $P<0.001$, $n=60$). Cohort biomass was computed as the sum of the biomass of the individuals recorded in all experimental quadrats and reported as g m^{-2}.

The production of each cohort was calculated by the Allen-curve method (Niell 1979, Cousens 1984). This is a graphical method that relates the number of individuals of a cohort (N) with their mean individual weight (w) at different times. After the maximum of abundance is reached (maximum recruitment), only mortality (a decline in density) and individual growth (an increase in mass) occur through the rest of the life cycle of each cohort. The standing stock biomass (B) of each cohort at a given time is defined by N • w under the curve, while the production (P) of the cohort can be computed as the integral under the curve. Standing stock, production and production to biomass ratio (P:B) were computed for each cohort and for the total population for different time intervals.

Results

Environmental variability

The upwelling dynamics in the study area were characterized by a period of positive values between March and August and negative values in autumn and winter months, except for December 2010 when the mean value was also positive (Fig. 3.1). Contrary to expectations, the average upwelling conditions had no effect on the properties of surface seawater when grouped by upwelling and downwelling

periods (Table 3.1). Besides, there were no significant differences between locations for any of the measured variables, and there were only significant differences in temperature and NO_3^-+NO_2^- between upwelling and downwelling periods (two way ANOVA for the effect of location and period, as fixed factors, and their interaction, Table 3.1). However, these differences are the opposite of those expected from the effect of upwelling, as average values of both temperature and NO_3^-+NO_2^- during upwelling conditions were lower than during downwelling conditions, suggesting a major role of continental water inputs at both locations.

Description of the macroalgal assemblages

The mean (±se) biomass of the initial undisturbed population was higher at Mera (643±514 g m^{-2}) than at Ría do Burgo site (415±97 g m^{-2}). Accordingly, size class distributions of the initial population showed a higher number of individuals at Mera, with a predominance of individuals longer than 30.5 cm (top left, Figs. 3.2 and 3.3). The mean (±se) biomass of all accompanying flora summed up to 54±47 and 45±21 g m^{-2} at Ría do Burgo and Mera respectively. *Ulva* sp. was quite abundant at both sites at the denudation time (October 2010). In Ría do Burgo, some individuals of *A. nodosum* were also present at the experimental quadrats, contributing to ca. 10% of total biomass.

Table 3.1. Mean and se values of temperature (t, °C), salinity (S), total nitrate (NO_3^-+NO_2^- , µM), ammonium (NH_4^+, µM), and phosphate (PO_4^{3-}, µM) measured at each site and grouped for periods of upwelling (n=7) and downwelling (n=6) following the mean values in Fig. 3.1. P: significance of differences between periods (two way ANOVA, *: P<0.05).

		Ría do Burgo		Mera		
Variable	period	mean	se	mean	se	P
t	upwelling	15.0	1.3	17.3	1.1	*
	downwelling	14.3	1.0	18.2	0.8	
S	upwelling	32.7	0.5	34.1	0.8	n.s.
	downwelling	28.0	3.9	33.7	1.5	
NO_3^-+NO_2^-	upwelling	18.24	5.63	7.74	2.43	*
	downwelling	38.98	13.08	14.85	2.74	
NH_4^+	upwelling	11.52	3.16	8.90	2.75	n.s.
	downwelling	21.22	4.70	19.47	3.02	
PO_4^{3-}	upwelling	1.74	0.27	1.92	0.26	n.s.
	downwelling	2.18	0.62	2.16	0.42	

During the sampling period, the accompanying flora reached maximum abundance in June 2011. Mera showed the highest species diversity, with *Corallina elongata*, *Osmundea pinatifida*, *Chondracanthus acicularis*, *Gelidium pusillum*, *Cladostephus spongiosus*, *Leathesia difformis*, and some species from the Order *Ceramiales* and *Ulva* species, especially *U. compressa* that were abundant from May until July. In contrast, only *G. pusillum*, *Caulacanthus ustulatus* and *Chaetomorpha aerea* were recorded at Ría do Burgo.

Size distributions

After the initial denudation of the experimental quadrats three cohorts were identified during the sampling period at each site (Figs. 3.2 and 3.3). However, the progress of the size class distributions of these cohorts was different at both sites. Ría do Burgo was characterized by its fast recovery after scrapping, while recovery at Mera was slower. The cohorts appeared in November 2010, and in March and June 2011 in Ría do Burgo, although new recruits had progressively joined up the population in previous months (Fig. 3.2). In Mera, the cohorts appeared in November 2010 and January and July 2011 (Fig. 3.3). Total and cohort abundance were always higher at Ría do Burgo than at Mera. The lowest recruitment was observed for the first cohort (November 2010) at Ría do Burgo and for the second cohort (January 2011) at Mera, while the highest recruitment was recorded for the third cohort in June and July 2011 at both sites respectively.

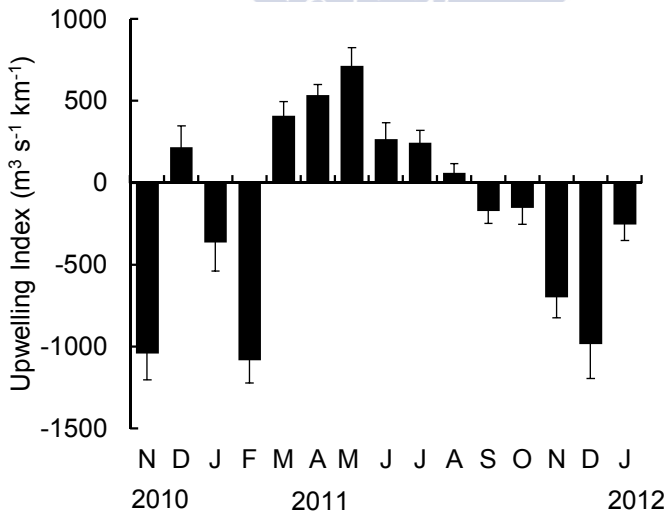

Figure 3.1. Monthly means (±se) of the upwelling index ($m^3\ s^{-1}\ km^{-1}$) computed in a grid of 1°•1° centred at 43°N, 11°W from November 2010 to January 2012.

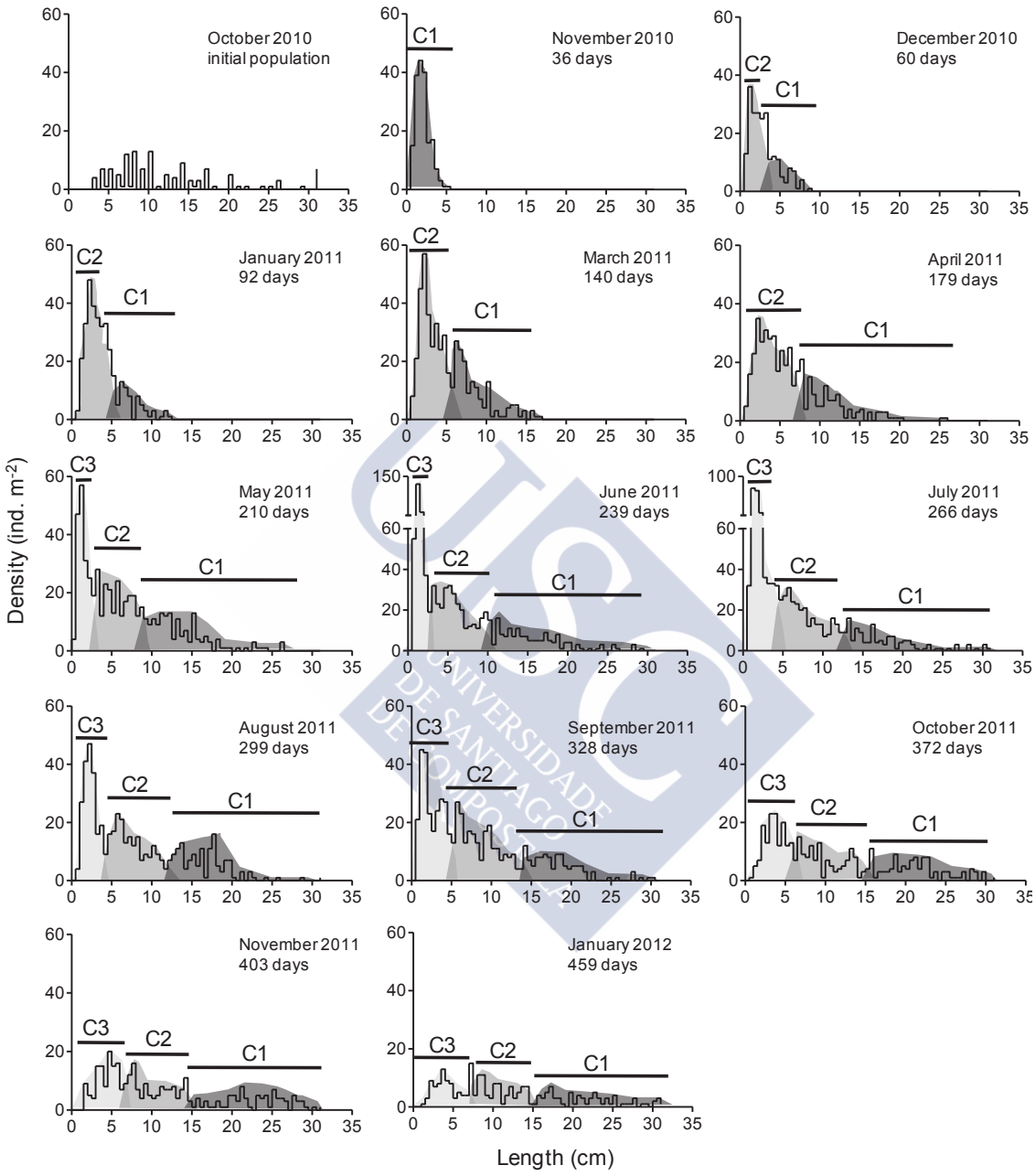

Figure 3.2. Temporal variation of the size-class frequency distributions of *F. vesiculosus* population at Ría do Burgo. Three cohorts (C1, C2 and C3) are identified. The number of days since the denudation date (October 2010) is specified on each histogram. The upper left histogram shows the size distribution of the scrapped individuals in the experimental quadrats in October 2010.

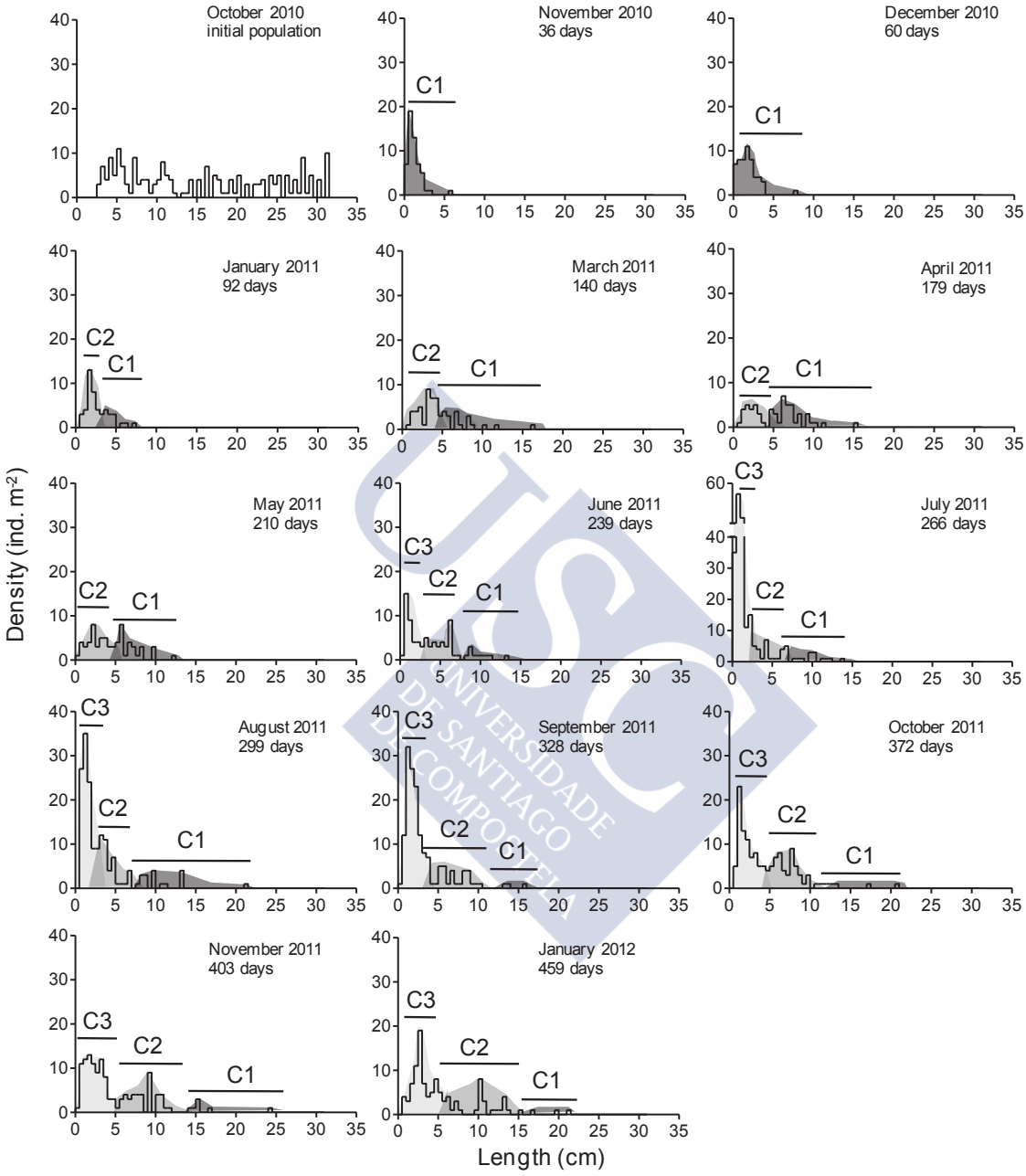

Figure 3.3. Temporal variation of the size-class frequency distributions of *F. vesiculosus* population at Mera. Three cohorts (C1, C2 and C3) are identified. The number of days since the denudation date (October 2010) is specified on each histogram. The upper left histogram shows the size distribution of the scrapped individuals in the experimental quadrats in October 2010.

In January 2012 (15 months after the denudation), the size distribution of Ría do Burgo was the most similar one to the initial undisturbed population (Fig. 3.2). On the contrary, by the same time, the size distribution of the population in Mera was skewed towards individuals <5 cm, very different from the initial undisturbed population (Fig. 3.3).

Growth

Individuals from the first cohort showed the fastest growth and maximum length, and individuals from the third cohort the lowest growth (Fig. 3.4), but in Mera, the growth rates were slower than at Ría do Burgo. At both sites, however, there was a relatively large variation in individual growth rates during the first year of life.

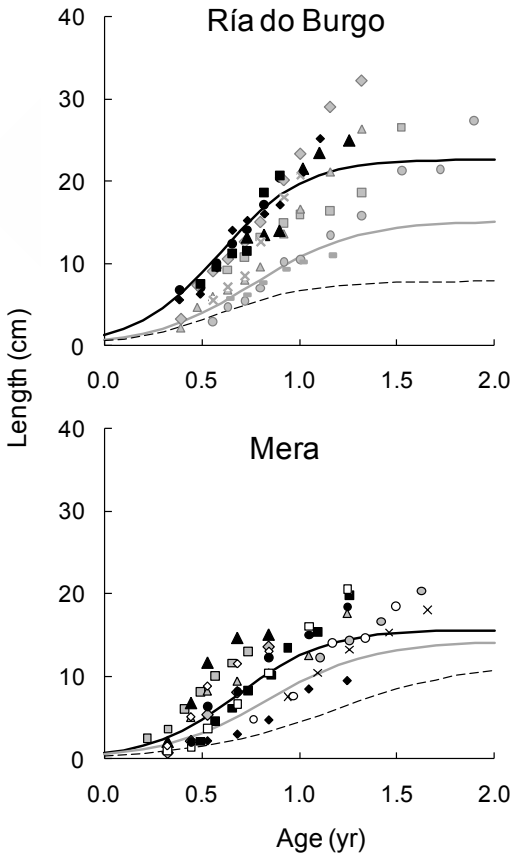

Figure 3.4. Variability of individual growth of labeled *F. vesiculosus* specimens at Ría do Burgo (top graph) compared with the modal growth curves of cohort 1 (black line, $L=22.6/(1+e^{(2.8-4.7\ Age)})$), cohort 2 (gray line, $L=15.1/(1+e^{(3.0-3.9\ Age)})$) and cohort 3 (dashed line, $L=7.85/(1+e^{(2.5-4.3\ Age)})$) and at Mera, compared with the modal growth curves of cohort 1 (black line, $L=15.6/(1+e^{(3.1-4.5\ Age)})$), cohort 2 (gray line, $L=14.3/(1+e^{(3.2-3.8\ Age)})$) and cohort 3 (dashed line, $L=11.7/(1+e^{(3.3-2.8\ Age)})$). For each site, sets of points of the same symbol represent the observed growth of one individual (n= 10 individuals at each site).

Reproductive structures

Some of the individuals, but particularly those of cohort 1 originated in November, showed reproductive tips after 6 months of life (Fig. 3.5). At that time, individuals with receptacles ranged from 8.5 to 25.5 cm long in Ría do Burgo, and from 9.5 to 15 cm long in Mera. The smallest individuals with receptacles were 7 cm (in September 2011, Ría do Burgo) and 6 cm long (in June 2011, Mera).

The highest proportion of reproductive individuals from the first cohort was observed in January 2012 and October 2011 at Ría do Burgo and Mera respectively (Fig. 3.5). However, only a few individuals of the second cohort showed receptacles at both sites, especially at Ría do Burgo, where they were absent until September 2011. In Mera, the highest percentage of reproductive individuals of the second cohort was observed in July 2011. No reproductive individuals from the third cohort were observed at Ría do Burgo while in Mera they did not appear until January 2012.

Survivorship and production

The population from Ría do Burgo displayed a pattern of increasing mortality rates and recruitment from the first (highest values) to the third cohort (lowest values), while the population from Mera showed similar mortality values for all cohorts and only relatively high recruitment for the third cohort (Fig. 3.6). In all cases, recruitment was higher at Ría do Burgo.

Despite the low recruitment, the first cohort of both populations was always the most productive because of their relatively high survival rates and their faster growth, as shown by the Allen-curves (Fig. 3.7). The net production values estimated from the areas under the curves were particularly high at Ría do Burgo, where accumulated production of cohort 1 exceeded 16 times that of cohort 3 and ca. 3 times the production of cohort 2 for the first 3 years of life (Table 3.2). At Ría do Burgo, the highest values of biomass and production were reached by all cohorts during the first year of life of the population. While in Mera, only the first cohort showed the same pattern, and cohorts 2 and 3 showed the highest biomass and production values during their second year of life. For both populations, most cohorts produced excess biomass over the standing stock and showed a turnover rate >1 during the first two years of life, except cohort 1 at Ría do Burgo that during the second year only produced an equivalent amount to the standing stock (P:B=1). Production would be minimal during the third year for all cohorts, even if some standing stock biomass is still present (Table 3.2).

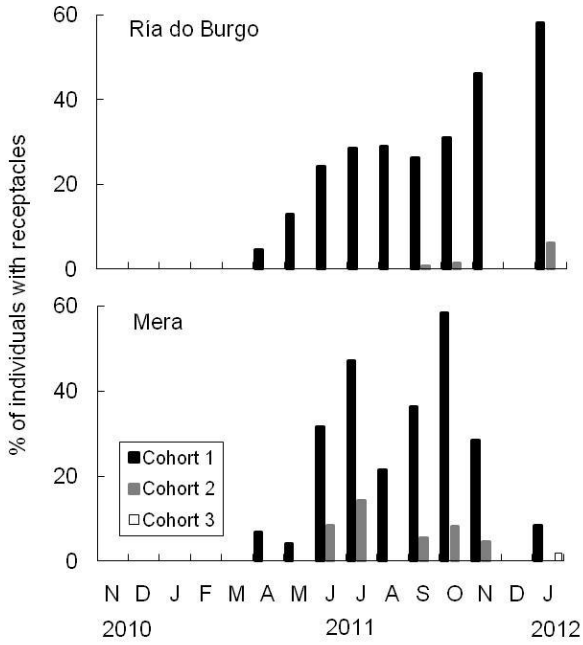

Figure 3.5. Percentage of individuals with receptacles of each cohort at each sampling site from November 2010 to January 2012. No fertile plants were found for cohort 3 at Ría do Burgo.

Discussion

The genotypic differentiation observed in fucoid species at their southern marginal limits of distribution suggest population´s different responses in their phenology due to changes in environmental variables (Billard et al. 2010, Zardi et al. 2013, Jueterbock et al. 2014). In this study, populations of *F. vesiculosus* showed lower growth, recruitment and survivorship rates compared to central populations. The low production rates measured imply that populations studied are very sensitive to habitat disturbances.

Growth rates and recruitment

Previous studies have highlighted the multiple factors influencing *Fucus* growth, i.e. temperature, nutrient concentration or wave exposure (Knight and Parke 1950, Mathieson et al. 1976, Chapman 1995, Bonsdorff and Nelson 1996), resulting in a variability that makes comparisons between sites and studies difficult. Even at the same site, there are differences among individual´s growth rates, as it was observed in

this study (Fig. 3.4). Variability among sampling sites has been attributed to external factors as temperature, salinity, or nutrient concentration (Steen and Rueness 2004) but also to intrinsic factors of the macroalga (Fuentes 1986, Ang 1991a, b). While external factors have been widely studied little is known about intrinsic factors affecting the growth of *F. vesiculosus*.

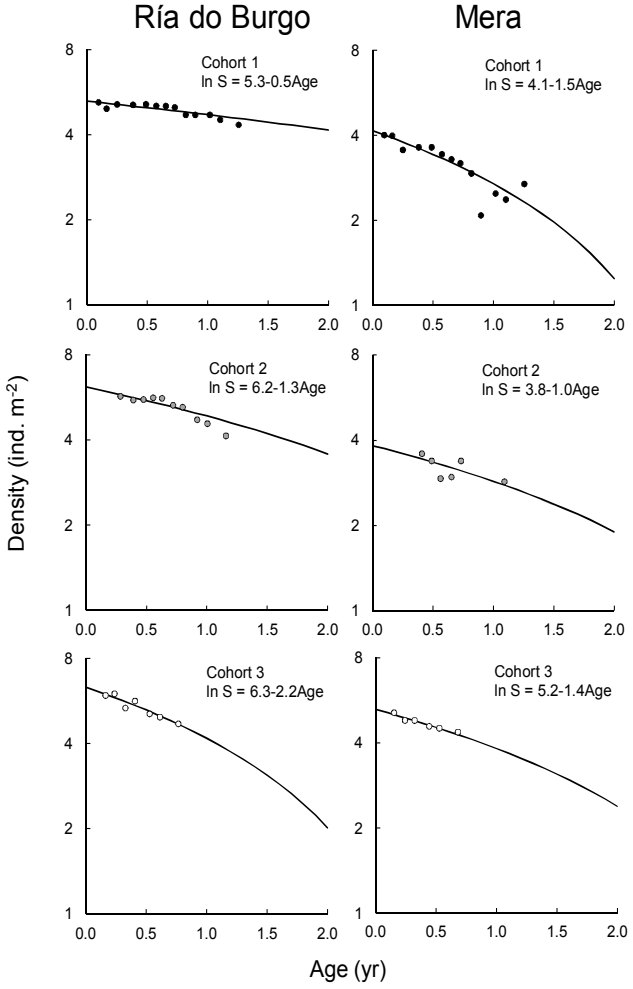

Figure 3.6. Ln survivorship curves of the three different cohorts at the two sampling sites. The corresponding exponential equations (r^2>0.9) are shown.

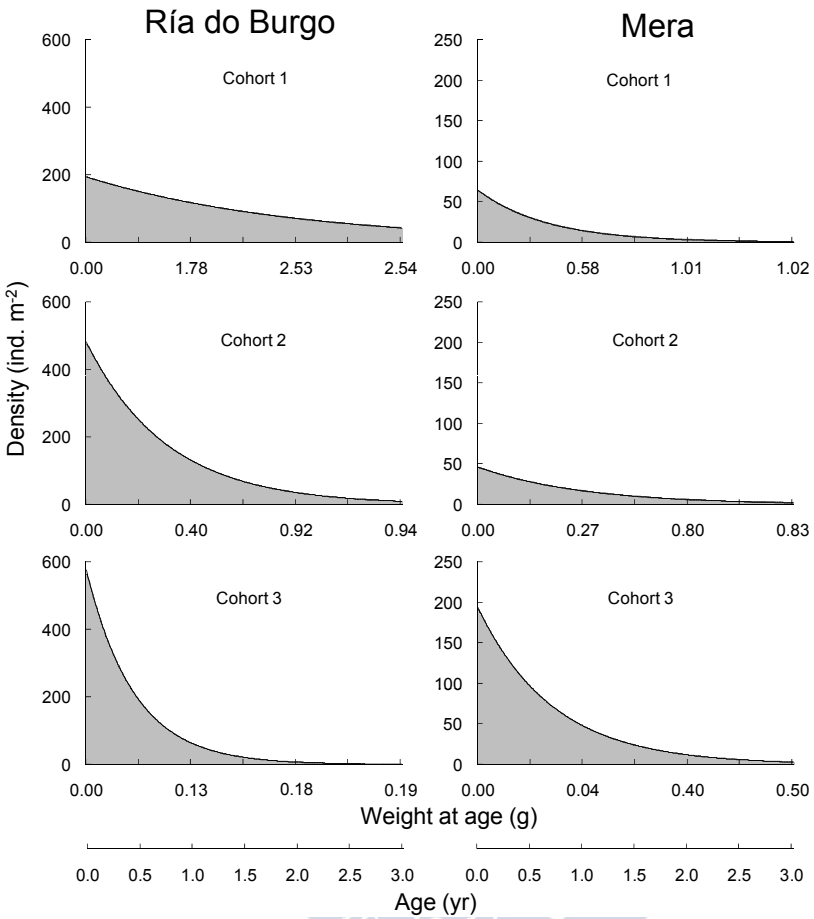

Figure 3.7. Allen-curves computed for each cohort at the sampling sites. Density of the individuals at each time was calculated using the survivorship equation in Fig. 3.6 and the individual weight at age (g) obtained from growth equations in Fig. 3.4 and the length-weight relationship. The age (years) corresponding to each weight was represented in a supplementary X-axis. Notice the different density scales for each site and the different weight scales for each cohort.

The growth in length of the studied populations followed a logistic pattern, as it was described for other Fucales (e.g. Niell 1979) but never before for this species. The growth rate reached a maximum when individuals were between 6 months and 1 year old, but stabilized thereafter. The length of old individuals can even decrease due to breakages after gamete release or apical damage and it has been observed that individuals of *F. vesiculosus* can easily regenerate new fronds from the holdfast after destructive events (Åberg 1989, Malm and Kautsky 2004). These results agree with the reports of significant differences in growth rates between size classes of *F. vesiculosus* (Fuentes 1986) and other *Fucus* species (Ang 1991b), with maximum rates in the smaller sizes.

Table 3.2. Standing stock biomass (B, g dry weight m^{-2}), production (P, g dry weight m^{-2} $period^{-1}$), and P:B ratio ($period^{-1}$) computed for the three cohorts observed at each site for different age periods (years).

	Ría do Burgo								
	Cohort 1			Cohort 2			Cohort 3		
Age (yr)	B	P	P:B	B	P	P:B	B	P	P:B
0.0-0.5	37.64	39.93	1.06	8.49	10.06	1.19	4.05	5.59	1.38
0.5-1.0	211.27	204.73	0.97	52.84	62.82	1.19	8.12	11.81	1.45
1.0-2.0	179.87	76.28	0.42	32.63	46.64	1.43	1.30	2.29	1.76
2.0-3.0	109.46	0.54	0.01	9.07	0.49	0.05	0.15	0.01	0.03
0.0-3.0	-	321.49	-	-	120.01	-	-	19.69	-

	Mera								
	Cohort 1			Cohort 2			Cohort 3		
Age (yr)	B	P	P:B	B	P	P:B	B	P	P:B
0.0-0.5	1.57	1.88	1.19	0.52	0.59	1.14	0.32	0.40	1.26
0.5-1.0	8.36	10.56	1.26	4.77	5.35	1.12	2.17	2.56	1.18
1.0-2.0	3.19	4.21	1.32	4.99	6.46	1.29	4.63	8.65	1.87
2.0-3.0	0.72	0.02	0.02	1.89	0.12	0.06	1.42	0.77	0.54
0.0-3.0	-	16.66	-	-	12.62	-	-	12.37	-

However, the direct comparison of growth rates from different studies is difficult because only maximum or average increases in length per unit of time, but without reference to the age or initial length of the individuals, are reported (Knight and Parke 1950, Fuentes 1986, Bonsdorff and Nelson 1996). From our results, the growth in length expressed as a function of age is markedly nonlinear, implying that growth rates vary continuously from quasi exponential increases during the first months of colonization to almost no increase after the first year. Still, some comparisons can be made by assuming that maximum growth rates reported in other studies are similar to the exponential growth increases averaged over the first year of life in this study. We found increases of up to 2 cm mo^{-1} that are close to those reported in other locations in Galicia (Fuentes 1986) while values of up to 3 cm mo^{-1} were reported for French (Lemoine 1913) and British Isles populations (Knight and Parke 1950).

Differences among cohorts were also clear in terms of growth rates at both sites. Maximum growth rates were observed for the first cohort (1.8 and 1.3 cm mo^{-1} in Ría do Burgo and Mera respectively) while individuals from the third cohort were

the ones with the lowest growth rates (0.6 cm mo^{-1} at both sites, Fig. 3.4). Based on previous studies, several factors might influence these differences. The time when the different cohorts initially grew vary. Individuals from the first cohort started their exponential growth at spring time, one of the periods of maximum growth of this species at this latitude (Fuentes 1986, Lamela-Silvarrey et al. 2012). On the contrary, individuals from the third cohort started their exponential growth phase in autumn, when conditions for growth are suboptimal. Besides, the presence of a denser and longer canopy of individuals from the previously settled cohorts would have reduced the availability of nutrients and light for smaller individuals of the second and third cohort due to intraspecific competition. This is supported by the absence of any new cohort after the first 9 months of the study, when the three cohorts were observed. In contrast to what it was observed with growth of germlings, the presence of a canopy from the previously settled cohorts seemed to be a good environment soon after settlement, as maximum recruitment was observed in the third cohort at both sites (Figs. 3.2 and 3.3). In this case, the presence of longer individuals might be beneficial for zygote implantation, as this coverage protects the individuals from desiccation, grazers or sedimentation, the major factors that influence survivorship during this first stage (Vadas et al. 1992). The absence of this initial coverage might also be related with the high mortality observed in the cohort 1 at Ría do Burgo (Fig. 3.6).

Differences between sites in terms of recruitment and growth rates were also found for all cohorts in our study, although results may be taken with caution due to all factors influencing both sites, apart from the ones considered. As seawater conditions were not very different among sites (Table 3.1), other reasons as the differential wave exposure or the spatial distribution of the populations may be partly responsible of the differences between sites. The population in the scraped area at the semi-exposed conditions of Mera was not protected by other macroalgal species due to the patchy distribution of this species in this location; and individuals from the first cohort were barely 5 cm long when the second appeared (Fig. 3.3). In contrast, the protected conditions at Ría do Burgo would facilitate the implantation and growth of new recruits as the experimental quadrats were surrounded by a dense population of *Fucus* but also by other large macroalgae (e.g. *A. nodosum*). This hypothesis might be supported by the differences found in growth rates related to the degree of wave exposure in other studies, with the result of maximum growth rates and sizes in estuaries, and minimum rates at open ocean locations (Knight and Parke 1950, Kalvas and Kautsky 1993, Bonsdorff and Nelson 1996). However, this is just a hypothesis as other authors

have observed that individuals at oceanic influenced sites were longer than individuals at estuarine locations sampled in the same ria although no mention is made to the growth rates or exposure conditions in these studies (Pazó and Romarís 1979).

Life span and reproduction

A large number of individuals of *F. vesiculosus* were able to survive through the 17 months of the present study and healthy populations remained in the experimental quadrats (Figs. 3.2 and 3.3). These results agree with the life span of 2-3 yr reported in many studies for most *Fucus* species, including *F. distichus* (Sideman and Mathieson 1983), *F. vesiculosus* (Knight and Parke 1950), *F. spiralis* (Niemeck and Mathieson 1976) and *F. serratus* (Knight and Parke 1950).

The studied adult populations might have a large reproductive potential (i.e. zygote production, Ang 1991a) as recruitment of the denudated surfaces occurred only one month after removal of adult plants. The identification of different cohorts through the year suggests continuous release of gametes, or at least that this release lasts for a long period of time, as observed in other *Fucus* populations (Knight and Parke 1950, Keser and Larson 1984a).

The reproduction of individuals growing within the experimental quadrats also appeared as a continuous process, as individuals with receptacles were always observed and its proportion in the populations increased through the period of observation (Ría do Burgo) or at least until the first autumn (Mera). In contrast, marked bimodal patterns, i.e. two distinct reproductive peaks, along the year were described for *F. vesiculosus* from other locations (Knight and Parke 1950, Niemeck and Mathieson 1976, Carlson 1991, Berger et al. 2001). Indeed, a population studied in the years 1983-1984, located only 10 km from Mera in the nearby Ría de Ares, was reported to produce receptacles between autumn and early spring and to release most gametes in early summer (Fuentes 1986). Nevertheless, the presence of fertile individuals, as the ones with receptacles within the quadrats, would not necessarily imply that those receptacles are mature (Berger et al. 2001). Moreover, receptacles could appear in the same individual but in different tips, giving the impression of continuous reproduction at the level of the whole plant (Ang 1992).

For most *Fucus* species, receptacles were first observed in individuals from 4.5 to up to 20 cm long or from 7 months to up to 2 years old (Knight and Parke 1950,

Niemeck and Mathieson 1976, Ang 1991b). Such a large variability might be due to differences among growth rates in different sites, but cannot be directly related to the age of the individuals. Fuentes (1986) observed than the mean length of fertile individuals in the nearby Ría de Ares was 11 cm, although he also found receptacles in individuals 7 cm long, as in the present study.

The results provided by our study could suggest that either local factors (like nutrient availability and wave exposure) or decadal fluctuations in the regional oceanography influenced the length of the reproductive period of this species. Even when the annual range of water temperature values reported in both Fuentes (1986) and our study are coincident (13-21 °C) the intensity of the Galician upwelling has decreased significantly since early 1980s (Bode et al. 2011a). A decrease in upwelling implies a decrease in the supply of new nutrients to the surface, but the concentrations of most nutrients near A Coruña have not shown any significant trend in the last two decades (Bode et al. 2011a), suggesting a compensation of the loss of upwelling nutrients with an enhanced input of terrestrial (mostly anthropogenic) nutrients. Besides, the variables measured in the surface water do not support a major role of upwelling dynamics in the present study. Runoff is maximum during autumn and winter, as indicated by the low salinity values in Table 3.1, and may introduce significant amounts of nutrients from terrestrial sources. Therefore the long period of reproduction found in this study cannot be attributed to a general increase in nutrients, at least at a regional scale.

Ladah et al. (2003) reported that protection from waves neither explains reproductive success nor influence in fertilization. Therefore the low impact of waves is not the reason for the persistence of receptacles in the studied estuarine population through the year in contrast with the findings of Fuentes (1986) in a more exposed population.

Production

As expected from growth and recruitment results, the rank in production of the cohorts was led by the first cohort at both studied sites while the third cohort was generally the less productive for all time-periods considered (Table 3.2). Early implantation in fall allowed the increase of the standing stock of the first cohort with rapid individual growth in the spring despite the low recruitment. In contrast, a relatively high recruitment in summer did not warrant high production

because of the reduced growth rates and high mortality observed during winter for the second and third cohorts. Estimated total production reached its maximum when the cohort was from 0.5 to 2 years old, depending on the site considered (Table 3.2). At this age, the increment in biomass depends only on the growth of survivors, and the presence of other *Fucus* individuals results in a competition due to the limitation for light and nutrients.

In all cases, but particularly for the first and second cohort, the values of production estimated for Ría do Burgo largely exceeded those for Mera, supporting the hypothesis that growth and survivorship are higher in wave protected, estuarine sites than at exposed, oceanic sites (Knight and Parke 1950). However, another study in Ría de Arousa (Galicia) reported lower production for estuarine sites than for oceanic sites (Fuentes 1986), suggesting that additional factors other than exposure and nutrients are also influencing local production.

All cohorts of the population studied at Ría do Burgo showed a lower turnover rate than those at Mera (Table 3.2). A larger turnover of biomass at exposed sites than at estuarine sites was also reported for other populations of *F. vesiculosus* in Galicia (Fuentes 1986) and was related to the enhanced energy flow in the former, favouring the access to nutrients and light in turbulent environments. For both sites, the production during the first year was almost equivalent to the standing stock biomass (P:B~1) indicating low renovation rates.

Mean annual production of the three cohorts in our study was much lower than those reported for *Fucus* species in other close sites but some decades ago (Table 3.3). The same reduction pattern in the production of fucoid species (including *F. vesiculosus*) was observed in the Cantabrian coast over the last 30 years (Lamela-Silvarrey et al. 2012). Although no direct causal mechanisms can be inferred to this fact, the general trend of increasing temperature due to climate change could be responsible (Lamela-Silvarrey et al. 2012). Still, comparisons of data from this study might be done with caution as methodological variations may in part explain this discrepancy. Previous production studies only consider average changes in biomass between consecutive sampling periods (generally 1 month apart) and our estimation is based on conservative modal values for growth and demographic parameters. Cousens (1984) has shown that differences in the assumptions when computing production using Allen curves in macroalgae may produce estimates varying by a

factor of 2, and even larger differences may result when considering only part of the annual cycle in the computations (Niell 1977a). Besides, in the present study the production was computed for a newly developed population after denudation, while in other studies the production is estimated for mature populations.

The low production rates estimated imply that the studied populations will recover slowly after denudation even when there were old plants remaining in the vicinity, particularly at the wave exposed site where the distribution of *F. vesiculosus* was patchy. Therefore these populations are sensitive to mechanical damage causing a loss in biomass and production not only in the dominant macroalgal cover but also in the accompanying flora and fauna.

Table 3.3. Production (g dry weight m^{-2} yr^{-1}) of *Fucus spiralis* and *F. vesiculosus* in the literature and this study.

Species	Production	Reference
F. spiralis	922.8	Niell 1977a
F. vesiculosus	979.9 - 1828.5	Fuentes 1986
	1431.8 - 2255.01	Lamela-Silvarrey et al. 2012
	41.65	Mera, this study
	461.79	Ría do Burgo, this study

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Growth and production of new recruits and adult individuals of Ascophyllum nodosum *in a non-harvested population at its southern limit (Galicia, NW Spain)**

Abstract

Populations near the geographic distribution limits of the species are considered to live under suboptimal conditions, and hence slight environmental changes can be critical for their survival. The potential sensitivity to disturbances of the long-living macroalga *Ascophyllum nodosum* was analyzed by the determination of growth, recruitment, mortality, and production of biomass of a population near its southern distribution limit. Recruitment, survival and growth rates of <2 yr old individuals were determined in a new population growing in experimentally denudated squares. Demographic data for >2 yr old individuals were obtained from individuals in the original population after estimating their age from the number of gas bladders in the thallus. Growth and survival were described as continuous nonlinear functions of age applied to the population and were further used to make demography-based production estimates. Recruitment of *A. nodosum* in denudated substrates seemed to require a previous cover of other macroalgae (as *Fucus vesiculosus*) as the only cohort detected during the 26-month period was observed after the *F. vesiculosus* individuals started to increase. The low production estimates (2,033 g m^{-2} for a 10-yr period) and poor recruitment may indicate a slow recuperation of this population to denudation. However, the large variability observed in the estimated growth curves of different populations along this southern distribution area suggests a large influence of local conditions that may help to overcome environmental changes at regional scales.

KEYWORDS: demography, growth curve, fucoid, size distribution, gas bladder, recruitment

* Viana IG, Fernández C, Bode A (in press) Growth and production of new recruits and adult individuals of *Ascophyllum nodosum* in a non-harvested population at its southern limit (Galicia, NW Spain). Mar Biol, doi: 10.1007/s00227-014-2553-0

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Introduction

In the last years an increasing attention has been focused on studying biogeographic differences among macroalgal populations along their distribution limits (Svensson et al. 2009, Araújo et al. 2014). Such studies have stressed that populations living near marginal areas of distribution are presumably constrained in their capacities, and that more variable and lower growth rates would be expected in edge populations compared to central populations (Angert 2009).

Consequently, changes at large spatial scales, like those associated with climate change, may have particularly high impact on these populations, and lower recovery capacities from disturbances are predicted to occur. Recent studies suggest that significant changes in brown seaweed populations are already occurring in temperate environments (Lima et al. 2007, Fernández 2011, Lamela-Silvarrey et al. 2012, NiCastro et al. 2013) supporting the importance of understanding the ecology of single species (Viana et al. in review b).

Ascophyllum nodosum (Linnaeus) Le Jolis is a dominant intertidal brown seaweed species, with its global distribution restricted to the North Atlantic Ocean. At the East coast, it is widely distributed from Northern Norway and the White Sea to the North of Portugal (Sharp 1987, Araújo et al. 2009, 2014). Present from sheltered to moderately exposed sites (Cousens 1982), populations usually cover moderately large areas of the rocky meso-littoral zone (Åberg 1992). This species can reach up to 2 m long as, even though it shows slow growth rate, it is one of the most long-lived macroalgae. Populations older than 15 years old have been described worldwide (David 1943, Niell 1979, Keser et al. 1981). Fronds have large egg-shaped gas bladders at intervals along the shoot that enable them to float during high tides. It presents apical growth (Moss 1970) and gas bladders are formed at the tip prior to the dichotomously branching that generally occurs once a year (MacFarlane 1932, Moss 1970). Receptacles are formed in lateral branches that could also be vegetative.

In central populations the distribution is almost continuous along the estuaries, and it is restricted by the wave action or substrata. These areas generally show high densities that support harvesting activities (Sharp 1987). They are also subject to sharp changes in environmental conditions, as ice covering, resulting in characteristic autoecological patterns (Strömgren 1986, Åberg 1992).

In marginal populations the distribution is more discontinuous than in northern areas, and the populations appear in isolated patches along the coast. These local populations are related with the seasonal inputs of cold water, as those caused by the spring-summer upwelling along the coast of NW Spain and N Portugal (Anadón and Niell 1981, Lüning 1990). Increasing sea surface temperature (SST) during the last decade has been related to changes in the distribution of several fucoid species (Lima et al. 2007, NiCastro et al. 2013), and although distributional shifts in *A. nodosum* southern limit were not observed (Lima et al. 2007), it appears that some populations have retreated in the Bay of Biscay (Alcock 2003).

A. nodosum populations show poor sexual reproduction success. Zygotes disperse short distances and suffer high mortality before and after settlement (Chapman 1995, Dudgeon et al. 2001). Thus, recruitment can be highly variable between years and established populations often consist on large, old individuals and relatively few recruits (Dudgeon and Petraitis 2005). Therefore, knowledge about the ecology of their early life stages and recruitment is important to estimate the possibilities of recuperation of these populations following a disturbance, as well as the conditions of mature populations. The sensitivity of *A. nodosum* populations to small and large-scale disturbances has been studied experimentally (Dudgeon and Petraitis 2001, Cervin et al. 2004, Araújo et al. 2009), but there is scarce information about the demography of populations under environmental stress (Araújo et al. 2014).

The objective of the present study was to quantify growth rates, recruitment, survivorship and production of a population of *A. nodosum* near the southern limit of distribution of this species to infer its sensitivity to disturbances. The study site is located at the Ría do Burgo (Galicia, NW Spain), included in a region under the influence of coastal upwelling. The growth in length is also estimated for other populations along the Galician coast as an indicator of the variability in the responses of this species at local scale.

Material and Methods

Study site

The coast of Galicia (NW Spain) is characterized by the presence of rias (tidal inlets) sustaining high levels of biological production due to seasonal upwelling (Arístegui et al. 2006). *A. nodosum* populations are restricted to the inner part of the rias in this

area (Niell 1977b). Besides the inflow of marine waters, the rias receive water and nutrient inputs from small rivers that drain into them (Bode et al. 2011b). This area is within the southern distribution limit of *A. nodosum* in the East Atlantic coast.

The study was conducted at Ría do Burgo (43°20'N, 8°22'W) (Fig. 4.1), the inner part of the Ría de A Coruña, which opens to the sea by a bay of 15.7 km^2. The Ría do Burgo has a mean depth of 10 m and a short salinity gradient with estuarine characteristics. It is in the mouth of the river Mero, a small river with a small drainage basin (385 km^2) and a mean annual flow of 6.6 m s^{-1} (Varela et al. 1994). *A. nodosum* forms here a dense population at the mid-upper intertidal zone of the littoral and constitutes the dominant species of this range (Bárbara et al. 1995). *Fucus spiralis* and *F. vesiculosus* are the dominant species at the upper and mid-lower intertidal zone respectively, although other Fucaceae, such as *F. serratus* or *Pelvetia canaliculata*, are also present in the area. The littoral at this site follows the typical zonation described for rias in this area (Niell 1977b).

During the first 15 months of the study period, salinity (±0.1, Practical Salinity Scale) and temperature (±0.1 °C) of surface water were measured *in situ* with a portable conductivity meter (YSI Model 30, YSI Inc. Yellow-Springs, Ohio, USA). Samples of surface water were also collected for further determination of dissolved nutrients (NO_3^-+NO_2^-, NH_4^+ and PO_4^{3-}) in the laboratory following Grashoff et al. (1983).

Size distributions and abundance of new recruits from experimentally denudated squares

In the *A. nodosum* dominant zone at Ría do Burgo (Fig. 4.1), three 50x50 cm experimental squares were randomly set up at 2-2.92 m relative to the Lowest Astronomic Tide (L.A.T.) trying to cover the vertical distribution of this species in the area. The size of each quadrat represents the minimum sampling area required for biomass and demographic studies in this region (Niell 1977b, 1979). Density and further estimations were made by combining all the results obtained in the three experimental quadrats. The experimental squares were denudated in October 2010. All macroalgal individuals constituting the original population inside the squares were removed as close to the ground as possible and the substrate was also cleaned with a metal brush to ensure that no small individuals or the holdfast of any adult individual could remain attached. No further treatment was applied to the substrate to preserve as much as possible the integrity of the existing microflora. The material

obtained from the scraping was transported to the laboratory in plastic bags. All removed individuals were measured and the total biomass of *A. nodosum* and accompanying flora was determined as wet and dry weight (±0.01 g). Dry weight was determined after drying in an oven (50 °C) until constant weight. All fronds from the same holdfast were considered as an individual.

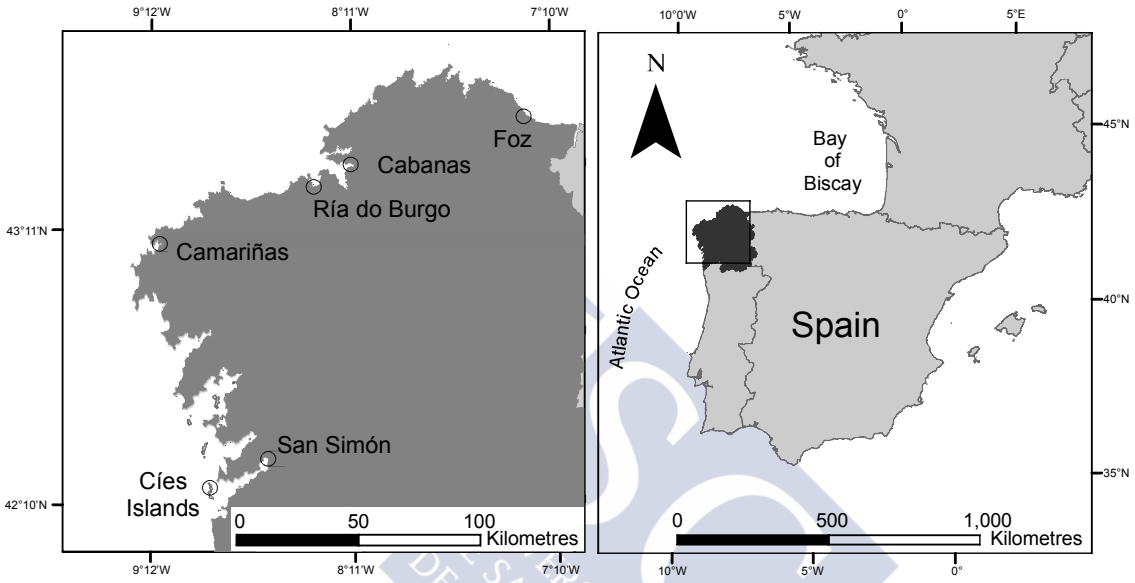

Figure 4.1. Location of study sites.

During a 26-month period (November 2010 to December 2012) all *A. nodosum* and *F. vesiculosus* individuals within each experimental square were counted and measured. From November 2010 to January 2012 the samplings were made every month, except in February and December 2011; and since January 2012 until December 2012 only *A. nodosum* individuals were measured bimonthly. To report individual growth, all individuals longer than 0.3 cm were mapped inside each square and measured (±0.2 cm) from the base of the holdfast to the tip. During early summer sampling surveys, when the abundance in some of the experimental squares was high, individuals shorter than 1.5 cm were counted and 90 of these individuals were measured to determine their mean length. All individuals recorded were classified in size classes, each size class corresponding to a 0.5-cm interval.

Monthly frequency distributions of size classes were calculated from the number of individuals in each size class. Cohorts, or individuals that share a particular event during a life span, i.e. a year class (Crisp 1971), were identified from the modes of the size-frequency distributions, assuming normal or log-normal distributions. Because recruitment (i.e. the number of new individuals appearing in the experimental squares) can occur during several months, we defined as age 0 of a cohort the first month that the mode of the distribution reached a distinguishing maximum density peak.

Growth and appearance of the annual gas bladders in new recruits and adult individuals

Due to the slow growth of this species, the growth was recorded following two approaches: first as the observed growth of new recruits within the experimental denudated areas and second as the estimated growth of adult individuals from the original adult population.

Within the experimental squares, the growth was monitored by monthly changes of the modal length of the size-class distributions. The changing position of the mode between consecutive sampling dates affords an approximate measure of the changing size (growth) of that cohort (Crisp 1971). The modal length of cohorts can be fitted to a logistic equation (Niell 1979):

$$L_t = L_{max} / (1 + e^{(a1 + a2\ Age)})$$

where L_t is the modal length at time *t*, L_{max} is the asymptotic length, and a1 and a2 constants. The regression parameters were estimated by iteration after an initial estimation of their value and subsequent minimization of the sum of squares by the Marquardt method. GraphPad Prism 4 software was used to estimate the parameters of the fitted curves.

The second approach was based on the estimation of the growth through the correlation of the length and the number of gas bladders of adult individuals from the original population. The determination of the minimum age of adult *A. nodosum* individuals can be done by assuming the annual production of one gas bladder (Baardseth 1955), and by establishing the appearance of the first gas bladder in the year class 2 (Niell 1979, Cousens 1982). Therefore, a growth curve can be back estimated if the length and number of gas bladders of different individuals in a

population are recorded at one moment in time. Following this method, the growth of *A. nodosum* was estimated from the original population sampled at Ría do Burgo in October 2010. Temporal and geographic variability in growth was analyzed after estimating growth curves from samples collected at Ría do Burgo in 2006 and at other sites along the coast of Galicia (Fig. 4.1). These estimated curves were also compared with the curves determined for Cíes Islands and San Simón by Niell (1979).

To test that the formerly assumptions were correct, the appearance of the annual gas bladder in new recruits and labeled adult individuals was checked. Two approaches were made, i) the percentage of new recruits within the experimental squares with the first gas bladder was recorded; and ii) seven adult individuals close to the experimental squares were labeled to follow the appearance of the annual gas bladder.

Demography and production of new recruits from the experimentally denudated squares

The fate of individuals within the experimental squares was monitored based on the monthly records of their position within each experimental square. New recruits were considered when they were first detected. Any individual that disappeared from the experimental squares was considered dead. Survivorship (S) was calculated as the fraction of individuals remaining from the cohort maximum recruitment and fitted to an exponential decay function with age:

$$S = S_0 \, e^{-(m \, Age)}$$

where S_0 is the density when the cohort showed maximum recruitment and m the mortality rate.

The dry weight biomass of each individual (w, g) was determined using a length-weight relationship computed from a set of individuals of different lengths (L, cm) and without receptacles collected at Ría do Burgo ($L=21.962w^{0.418}$, $r^2=0.962$, $P<0.001$, $n=42$). The total biomass of the population was computed as the sum of the biomass of the individuals recorded in all experimental quadrats and reported as g m^{-2}.

The production was calculated by the Allen-curve method (Niell 1979, Cousens 1984). Production estimates using this technique can be obtained graphically by relating the

number of living individuals of a cohort (N) and the mean weight of those survivors (w) at corresponding times (Crisp 1971). After the maximum of abundance is reached (maximum recruitment), only mortality (a decline in density) and individual growth (an increase in biomass) occur through the rest of the life cycle of the cohort. The standing stock biomass (B) of each cohort during a period of time is given by the square defined by N • w under the curve, while the production (P) of the cohort can be computed as the integral under the curve. Standing stock, production and production to biomass ratios (P:B) were computed for different time intervals.

Results

Environmental settings and characteristics of the experimental squares

The site showed large ranges of variation in both temperature and salinity (Fig.4.2). Temperature reached a maximum of ca. 23 °C by the end of summer while minimum values in winter reached 10 °C. Low salinity values were recorded during fall and winter. Nutrient concentrations also varied during the sampling period (Fig. 4.2). Nitrate plus nitrite and phosphate were maximum during winter months, while values in early summer were below 5 µM. The fact that the lowest values were found during upwelling months (spring and summer) and the inverse variation of salinity vs. nitrate plus nitrite suggests a major role of river nutrient inputs in this location.

The mean (±se) dry biomass of *A. nodosum* individuals removed from the original population within experimental squares was 3,456±1,241 g m^{-2}. Among the possible accompanying species, only *F. vesiculosus* was present within all the experimental squares in the original macroalgal assemblage, averaging 108±144 g m^{-2}. During the first months after denudation several specimens of *F. vesiculosus* appeared in the experimental squares, reaching higher densities than *A. nodosum* (Fig. 4.3). Nevertheless, other seasonal macroalgae were recorded during the sampling months, with the maximum number of species detected in June 2011. From May until July, when water temperature was high, *Chaetomorpha aerea* was present, and among Rhodophyceae, only *Gelidium pusillum* and *Caulacanthus ustulatus* were recorded.

Size distributions of new recruits from the experimentally denudated squares

After the denudation (October 2010), and during the period of study (~2 yr), new *A. nodosum* recruits were observed all along the sampling period. During the first months the recruitment of this species within the experimental squares was

minimum, and only up to 50 individuals m^{-2} were mapped until April 2011 (Figs. 4.3 and 4.4). In spring, when the number of *F. vesiculosus* individuals started to increase, the number of individuals of *A. nodosum* also started to rise, although at slower rates than *Fucus* (Fig. 4.3). In accordance, and based on the monthly variation of the size-distributions, the start of the only cohort detected during the study was identified in May 2011, when a marked density peak was observed (Fig. 4.4).

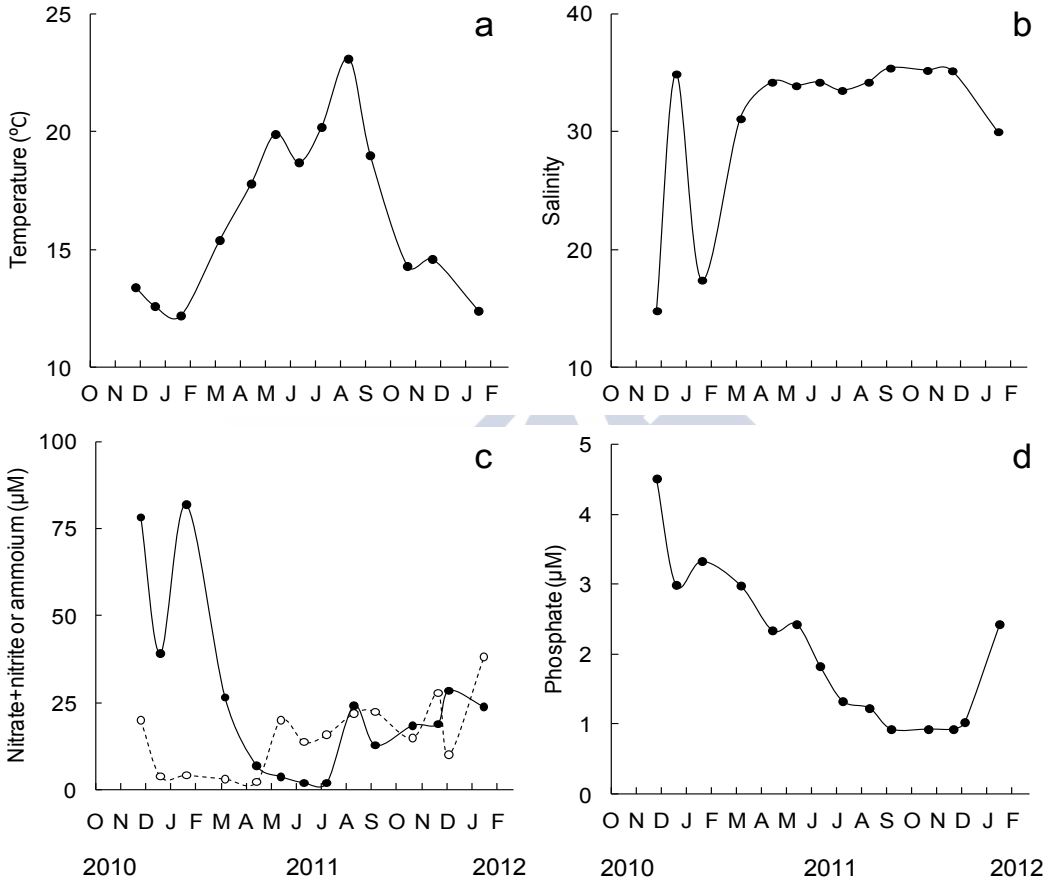

Figure 4.2. Variations in water temperature (a, °C), salinity (b), nitrate+nitrite and ammonium (c, µM, continuous and dashed lines respectively), and phosphate (d, µM) at Ría do Burgo site during the first 15 months of the study period.

Since June 2012 a few *A. nodosum* individuals longer than 12.5 cm were observed, but their abundance was very low compared to size classes shorter than 5 cm. And by the end of the sampling period (December 2012) the highest densities were still recorded among individuals shorter than 5 cm. Individuals between 12.5 and 26 cm (the maximum length observed) reached approximately 60 indiv m^{-2}, while in the original population most individuals were longer than 60 cm (Fig. 4.4).

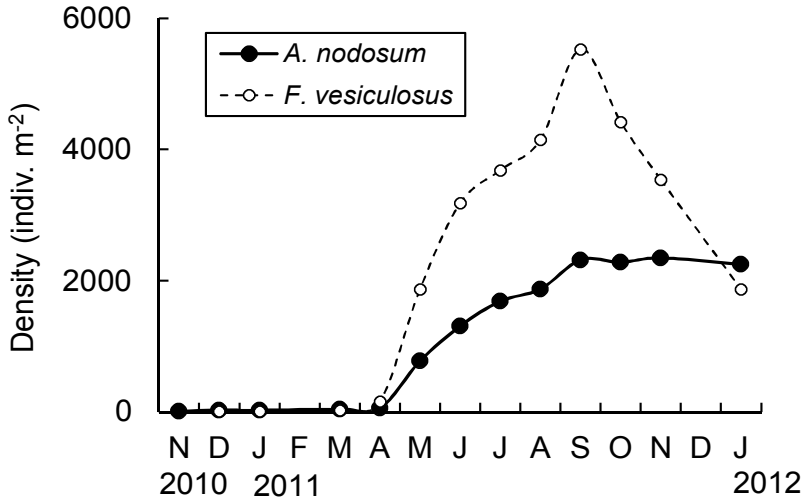

Figure 4.3. Variations in density (individuals m^{-2}) of the new recruits of *A. nodosum* and *F. vesiculosus* in the experimental squares at Ría do Burgo during the first 15 months of the study period.

Growth and appearance of the annual gas bladders in new recruits and adult individuals

The first gas bladder in the new recruits within the experimental squares appeared in March 2012 (Fig. 4.5). All individuals within the experimental squares that showed a gas bladder were between 7 and 18 cm long and were not more than 17 months old, the time since the denudation was done. But during the 26 months of the study not all recruits longer than 7 cm had a gas bladder.

Among adult labeled individuals, the annual gas bladder was present at the tip of all individuals in the spring of both recorded years. During the first spring (2011) the bladder was developed later (between March and April) than in the second year (January-March). The month after a new gas bladder was observed for the first time, it was not at the tip of the individual anymore, and in some individuals a bifurcation was already present following the formation of the gas bladder. This last feature was not conspicuous to all individuals, as some of them showed 2 vesicles in a row or even a bifurcation on the vesicle.

Although there were some individuals within the experimental squares exceeding 12 cm at the end of the study, the modal length of the new recruits was much lower (Fig. 4.4). To complete the estimated growth curve of Ría do Burgo, data from the individuals of the original population within the experimental squares were

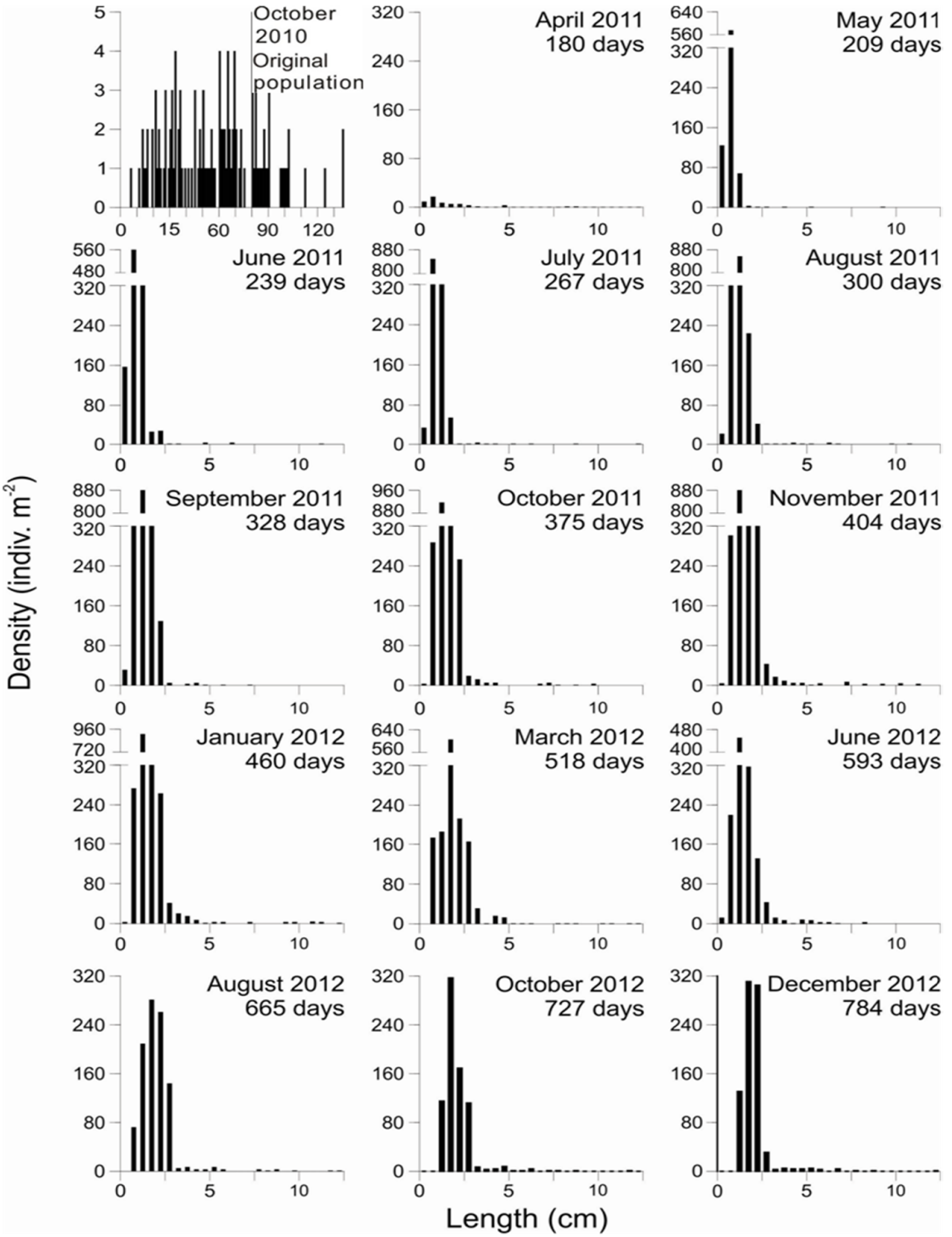

Figure 4.4. Variations in density (individuals m^{-2}) of new recruits of *A. nodosum* by 0.5-cm size-classes (<12.5 cm) in the experimental squares at Ría do Burgo during the study period. The size distribution of the original population before denudation is shown in the upper left panel. The time after denudation (days) along with the date of observation is also indicated on each histogram. Distributions shown are from April 2011 just before first density peak was detected. Note the different scales of the y- axis.

combined along with the modal length of the new recruits (Fig. 4.6a). The estimated growth curve showed slow growth during the first years of life and an exponential growth until the individuals were 95 cm long (Table 4.1).

The populations from other localities showed growth curves similar to those obtained at Ría do Burgo (Fig. 4.7a). The growth curve of Cíes Islands showed the slowest growth rate (Fig. 4.7a). There were no differences between the growth curves of Ría do Burgo at different sampling years; and for both years, the oldest individuals observed were 9 years old (Fig. 4.7b).

Survivorship and production of the new recruits within experimental squares

The survivorship was estimated from the date the maximum abundance of the cohort was detected. The new population displayed high recruitment (~2,500 indiv m^{-2}) but also high mortality among individuals younger than 2 years old (Fig. 4.6b). The estimated survivorship curve was completed with density values of older individuals (≥4 years) of the original population. The mortality among individuals older than 3 years old was estimated to be much lower.

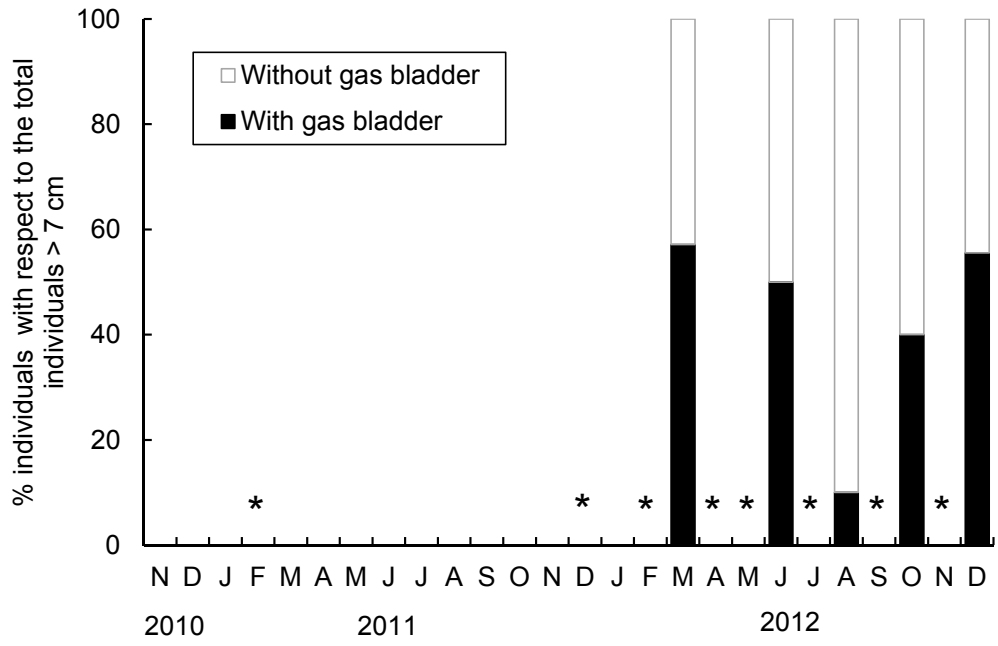

Figure 4.5. Record of the appearance of the annual gas bladders in new recruits longer than 7 cm and percentage of those new recruits with or without a gas bladder within the experimental squares at Ría do Burgo during the sampling period. *: No data.

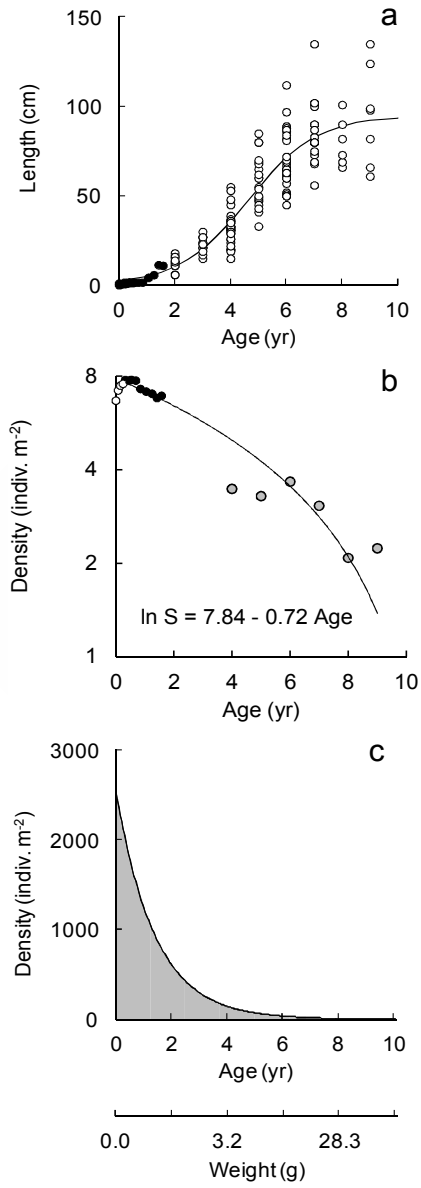

Figure 4.6. Growth in length (a, cm), ln survivorship (b, individuals m^{-2}) and Allen diagram (c) of *A. nodosum* population at Ría do Burgo. The open dots in panel **a** indicate adult individuals measured in the original population (2010) dated using the number of gas bladders and length. The black dots in panel **a** indicate the modal length of new recruits observed during the sampling period (Fig. 4.4). The parameters of the estimated growth curve in panel **a** are listed in Table 4.1. In panel **b**, the black dots indicate the density of new recruits observed during the sampling period (Fig. 4.4), the open dots are density estimations of the new recruits not used in the computation of the survivorship curve and the gray dots represent the density estimations of adult individuals from the original population (2010).

Table 4.1. Parameters of the logistic equations describing growth in length (cm) of *A. nodosum* computed for different sites along the Galician coast (see Figs. 4.1 and 4.7). The equation is in the form: Length = L_{max} / (1+ $e^{[a1+a2\ Age]}$), where L_{max} is the asymptotic length (cm) and age is measured in years. a1 and a2 are constants; n: number of data; r^2: determination coefficient.

	Sampling year	Latitude N	Longitude W	L_{max}	a1	a2	n	r^2
Foz	2010	43.56468	-7.24599	135.90	3.61	-0.55	42	0.88
Cabanas	2010	43.41146	-8.17255	113.50	3.21	-0.58	46	0.79
Ría do Burgo	2010	43.32777	-8.07357	95.09	3.70	-0.80	133	0.77
Ría do Burgo	2006	43.32777	-8.07357	101.40	3.21	-0.61	37	0.80
Camariñas	2007	43.13694	-9.17705	97.07	3.40	-0.90	25	0.86
San Simón *	1978	42.30645	-8.62840	100.00	2.27	-0.43	-	0.82
Cíes Islands *	1976	42.22461	-8.90689	105.65	2.40	-0.18	-	0.90

* from Niell (1979)

Despite the low recruitment, individuals between 2 and 6 years old were the most productive (Table 4.2). This is due to the higher standing stock biomass of the individuals at these ages because of the higher growth rates (Fig. 4.6a). Individuals between 8 and 10 years old may suffer several breakages, diminishing the standing stock biomass of the aged population, and in consequence reducing its production. The P:B ratio also supports that the biomass produced annually is over the standing stock (P:B>1) until the population is between 6 and 8 years old.

Table 4.2. Standing stock biomass (B, g m^{-2}), production (P, g m^{-2} $period^{-1}$) and P:B ratio ($period^{-1}$) estimated for the population of *A. nodosum* at Ría do Burgo using the Allen method for different time periods (yr).

Age (yr)	B (g m^{-2})	P (g m^{-2} $period^{-1}$)	P:B ($period^{-1}$)
0-1	33.52	46.95	1.40
1-2	96.42	104.16	1.08
2-4	455.99	708.66	1.55
4-6	568.68	925.00	1.63
6-8	231.04	229.75	0.99
8-10	62.19	18.43	0.30
0-10		2032.96	

Discussion

The studied population of *A. nodosum* showed low recruitment, growth, survival and productivity rates. These results are consistent with low recovery capacity associated to environmental stress at the southern limit of distribution of this species (Araújo et al. 2014). However, the local variability observed in the estimated growth curves suggests the existence of different local life history traits that may enhance the survival of this species under stress.

Recruitment

The slow recovery of *A. nodosum* following natural or experimental denudation was previously reported in natural and harvested areas (Knight and Parke 1950, Baardseth 1970, Vadas et al. 1990, Jenkins et al. 1999, Cervin et al. 2005, Ingólfsson and Hawkins 2008). At the experimental squares of Ría do Burgo, new *A. nodosum* recruits were almost absent during the first six months of the study (Figs. 4.3 and 4.4) when most individuals outside the squares did not have receptacles (pers. obs.). The start of the cohort was established in May 2011, when the first peak (mode) was observed, in agreement with the presence of mature receptacles in March that were reabsorbed in April after releasing the gametes. Nevertheless, increasing densities were observed the subsequent months after the cohort was established. The existence of a bank of gametes that gradually developed could have originated the subsequent increase in density of the cohort months later it was established. However, recruitment may not be simply related with the reproduction of adult individuals. For instance, a peak in recruitment was not observed in spring 2012 (Fig. 4.4), likely because the already high density of plants within the experimental squares. They may have prevented the settlement of significant numbers of zygotes or at least their survival until they were first detected because of the intraspecific competition for space and resources.

Within the experimental squares, the rock was initially colonized by *F. vesiculosus* individuals, even though the biomass of this species was 32 times lower than *A. nodosum* biomass in the original population. The initial appearance of *Fucus* spp. individuals in denudated areas was also observed in northern areas (Keser and Larson 1984b, Jenkins et al. 1999, Dudgeon and Petraitis 2001, Cervin et al. 2005). Soon after settlement, a dense canopy seems to be a good environment that enhances survivorship of several macroalgal germlings (Cervin et al. 2005, Choi and Norton

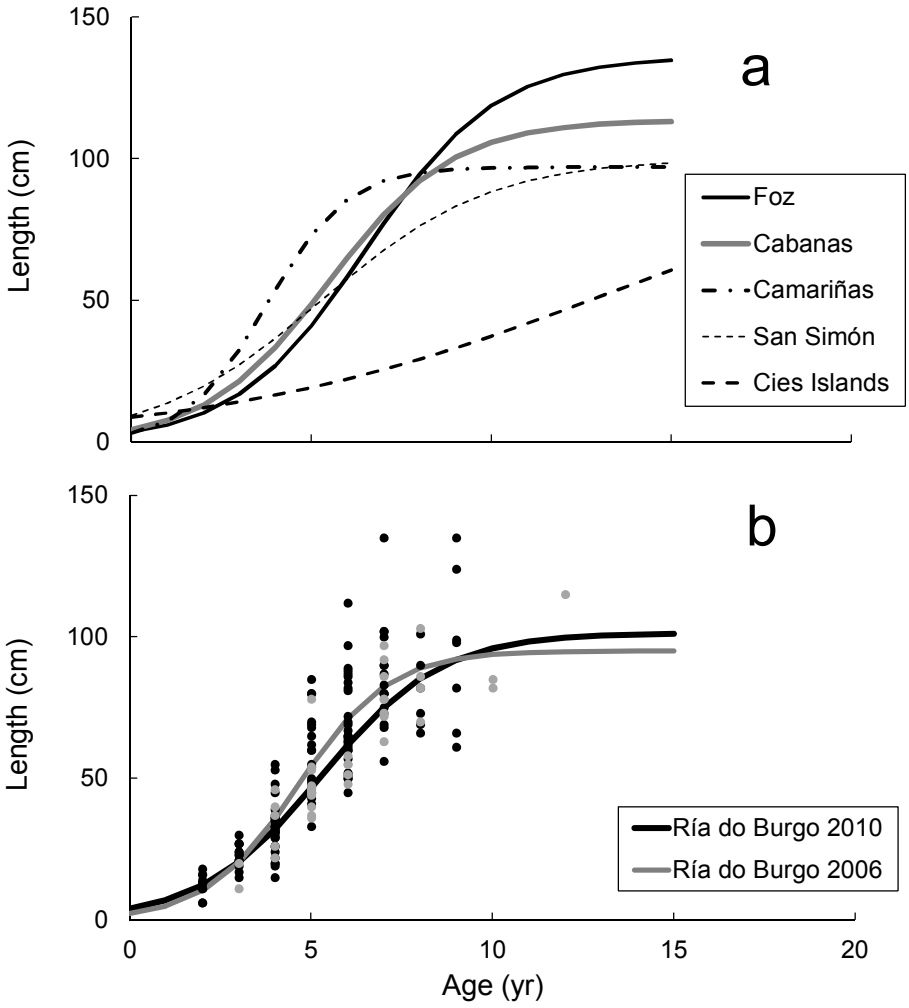

Figure 4.7. Estimated growth in length (cm) of populations of *A. nodosum* at several locations in Galicia (a) and at Ría do Burgo for years 2006 and 2010 (b). The parameters of the logistic equations are given in Table 4.1. The curves of San Simón and Cíes Islands are from Niell (1979).

2005, Sánchez and Fernández 2006), although later on, high densities may derive in a inhibition of growth rates due to the competition for nutrients, light, space, wave action or due to sweeping by large individuals (Vadas et al. 1992, Creed et al. 1996, Viejo et al. 1999, Steen and Rueness 2004, Cervin et al. 2005, Choi and Norton 2005).

Densities of new recruits observed in the present study (up to 2,500 indiv m^{-2}) were much lower than those reported for central populations after denudation,

where they can reach up to 700,000 indiv m^{-2} (Dudgeon and Petraitis 2001). On the other side, the density of the original adult population (160 indiv m^{-2}) were also low when compared to those found in central populations, where densities of adult individuals can reach up to 521 indiv m^{-2} (Keser et al. 1981). This suggests that environmental conditions in the populations near its southern limit can be suboptimal for recruitment, as supported by the lower reproductive value of *A. nodosum* populations in Portugal compared to populations in France due to the lower probability of transition to the class with the higher fertility (Araújo et al. 2014).

Growth of young and adult individuals

Most of the reported growth rates of *A. nodosum* were obtained from adult individuals (Mathieson et al. 1976, Stengel and Dring 1997) while studies recording the growth of young individuals are scarce (Vadas and Keser 1972, Schonbeck and Norton 1980). In this study, growth rates varied between 0.2-0.6 cm mo^{-1} during the first 1.5 yr since the start of the cohort (Fig. 4.6a). These rates were slightly lower than the average value of 0.8 cm mo^{-1} reported for young individuals growing in central populations (Cervin et al. 2005). For adults, growth increased exponentially after the second year of life, with maximum growth rates of 18.7 cm yr^{-1} for individuals between 5 and 7 years old (Fig. 4.6a). The maximum growth rates estimated in this study for other populations in Galicia varied between 5.5 to 21.3 cm yr^{-1} (Fig. 4.7a). In general, these values agree with reported growth rates in other regions (Mathieson et al. 1976, Peckol et al. 1988, Stengel and Dring 1997).

The studied population at Ría do Burgo showed a similar timing in the appearance of the first gas bladder to other populations (Baarsdeth 1955, Keser and Larson 1984b). This further supports the use of the number of gas bladders to calculate the approximate age of an individual of this species, although with a maximum error of ±2 yr (Niell 1979, Cousens 1984). Because the appearance of the first gas bladder occurs sometime between the first and second year, the growth curve estimated only from gas bladders data had a large uncertainty in the estimation of growth for individuals <2 yr old. To our knowledge, the present study is the first providing a growth curve based on direct measurements through the life time of *A. nodosum*, from newly implanted to old individuals. The length-at-age results of the direct length measurements for individuals younger than 2 yr old fitted along with those estimated for older individuals using gas bladders.

This estimation provides a continuous function of growth for populations of this species with reduced uncertainty at young ages.

Net growth in length for mature individuals is reduced, particularly for those >5 yr old, because of breakages of the thallus, even leading to shortening in the maximum length (Keser et al. 1981, Araújo et al. 2014). This effect is represented in the logistic growth curve by the plateau or maximum length (L_{max}) which is characteristic of each population and indicative of the local environmental conditions for sustaining a healthy adult population. Large values of L_{max} can result from the combination of high growth rates (i.e. enough nutrients and light) and small losses of biomass (e.g. by breakages), and are generally associated with eutrophic areas with low wave and current velocities (Niell 1977b). Thus, the analysis of the logistic growth curve parameters of different populations can be used for estimating life history traits and adaptation to local conditions. For instance, the variety of estimated local growth curves found in Galicia (Table 4.1, Fig. 4.7) is consistent with the large susceptibility to changes in individual length reported for southern populations of this species (Araújo et al. 2014). Populations with fast growth of young individuals (high a1) and reduced breakage with age (high a2) will reach high asymptotic length (L_{max}), as observed at Foz (Table 4.1). These populations will be less sensitive to extinction than those with low growth rates and high risk of breakage (e.g. San Simón, Table 4.1).

Production

The estimated produced biomass of one cohort at Ría do Burgo after a 10-year period (2,033 g m^{-2}, Table 4.2) was close to the range of variation of the biomass of the original population (2,215-4,697 g m^{-2}). This suggests that this value is not severely biased by the inclusion of a new starting population after denudation in the calculations. However, the overall productivity estimated in this study was below the estimated values for the same species at other sites in Galicia using only demographic data estimated from natural and mature populations. For instance, Niell (1979) estimated accumulated production values of 5,000 g m^{-2} for a 10-year period at Cíes and almost the same value only for a 7-year period at San Simón, but the populations at these localities had larger densities and lower mortality rates than the studied population at Ría do Burgo. Reported values of standing stock biomass in areas above 50°N reach up to 11.3 kg m^{-2} (Cousens 1984, Åberg 1992) but only up to 5 kg m^{-2} in other sites close to the study area (Niell and Soneira 1976, Cremades et al. 2004, Lamela-Silvarrey et al. 2012). In all cases, the maximum production is observed for

individuals of more than 4 years of life (Niell 1979, Cousens 1984), and in our study when the individuals are 4-6 years old (maximum P:B value in Table 4.2).

As found for recruitment and mortality, the low estimated productivity for the studied population at Ría do Burgo implies a high risk of failure when recovering from disturbances. However, the large range of productivity of this species in Galicia, and the local differences in growth suggest that the persistence of *A. nodosum* in the southern distribution limit would be mainly determined by the adaptation of populations to local conditions, while changes in regional climate would be of secondary importance. Adaptation of populations to local conditions may also explain that some populations of the N Spain have disappeared (as in Asturias) while others in close areas (as in Cantabria) remain (Alcock 2003). This conclusion is also supported by demographic studies showing higher adaptability of populations in Portugal compared to those in France (Araújo et al. 2014). Future studies of *A. nodosum* populations will benefit from determining key demographic parameters (as individual growth and Allen curves in this study) as indicators of local adaptation and sensitivity to disturbances.

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Experimental assessment of the macroalgae Ascophyllum nodosum *and* Fucus vesiculosus *for monitoring N sources at different time-scales using stable isotope composition**

Abstract

Stable isotope composition of brown macroalgae has been widely used to monitor N loading during the last decades but some of the required assumptions when using them to detect anthropogenic inputs remain untested. In this study several experiments were run with two key species, *Ascophyllum nodosum* and *Fucus vesiculosus*, to determine internal nitrogen isotope dynamics. First, the equilibration of the isotopic values of the different parts of the thallus of these species was tested by growing them under different water sources. Then, nitrate uptake capacity and N transport along the frond were tested by ^{15}N enrichment experiments. The results indicate that although the growing tips had the highest uptake rates, older parts of the frond of both species have the capacity to incorporate N at low rates. No evidence of N transport along the thallus, from the tip to the basal segment of the frond or the converse, was found. These results show that the growing tips of these macroalgae can be used to monitor N loadings at time scales from weeks (*F. vesiculosus*) to months (*A. nodosum*). The use of non-growing parts of the thallus to do retrospective studies cannot be recommended because of their measurable exchange of N with the surrounding water.

KEYWORDS: stable isotopes, enrichment, growth rate, Phaeophyceae, DIN

* Viana IG, Bode A, Bartholomew M, Valiela I (in review) Experimental assessment of the macroalgae *Ascophyllum nodosum* and *Fucus vesiculosus* for monitoring N sources at different time-scales u.sing stable isotope composition. J Exp Mar Biol Ecol

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Introduction

Concern with coastal eutrophication has increased in the last decades due to increasing N loading associated with the growing human population in these areas. Numerous methods have been developed to identify and quantify the N sources. Among them, the ratio of the stable isotopes of N ($\delta^{15}N$) in macroalgal tissues has been increasingly used. The $\delta^{15}N$ not only allows detecting the presence of anthropogenic N that is actually available for macroalgae in coastal water, but also allows estimating the intensity of the effluents and detect disturbances before alteration in structure and function occur in the ecosystem (McClelland et al. 1997, McClelland and Valiela 1998a, 1998b; Costanzo et al. 2001, Gartner et al. 2002, García-Sanz et al. 2010, 2011; Carballeira et al. 2013).

Among macroalgae, Phaeophyceae have been widely used for monitoring loads of N and other substances (e.g. heavy metals) (Viana et al. 2010, 2011). They are good biomonitors because they are perennial, resistant to pollution and accumulate dissolved substances in their tissues (Phillips and Segar 1986). They take up, assimilate and accumulate N in excess to growth demands so they can be used as integrators of N availability (Hanisak 1983), offering time-integrated measures of the exposure time. The Fucaceae *Fucus vesiculosus* and *Ascophyllum nodosum* are two long-lived canopy-forming macroalgae widely distributed at both sides of the Atlantic Ocean. They can live up to 15 yr in the case of *A. nodosum* (Niell 1979). Both of them show apical growth, so if growth rates are known (Viana et al. in review b, in press), different segments along the frond can be related with particular environmental or water conditions (Savage and Elmgren 2004, Raimonet et al. 2013, Carballeira et al. 2014). Moreover, *A. nodosum* fronds develop a gas bladder in the tip that generally occurs once a year (David 1943, Viana et al. in press). This annual bladder enables to estimate the minimum age of an individual and delimitate its annual growth (Niell 1979, Viana et al. in press). Both species of macroalgae have been previously used to monitor N sources (Hobbie et al. 1990, Savage and Elmgren 2004, Deutsch and Voss 2006, Bode et al. 2011b, 2014; Viana et al. 2011, Raimonet et al. 2013, Viana and Bode 2013, Carballeira et al. 2014).

The use of stable isotopes on macroalgae to detect N loadings requires some assumptions related to their ecology and physiology. For instance, the accumulation of heavy isotopes depends on the preference of light isotopes (or fractionation)

during uptake, and on the uptake capacity of tissues. Concentration dependent fractionation during uptake has been reported for some algae, as diatoms (Wada and Hattori 1978, Pennock et al. 1996). However, experimental studies in different macroalgal species, as the Chlorophycea *Ulva* (formerly *Enteromorpha*) *intestinalis* (Cohen and Fong 2005) and *Ulva pertusa* (Dudley et al. 2010) or the Phaeophycea *Cystoseira mediterranea* (García-Sanz 2009) demonstrated that, at least those macroalgae, did not exhibit concentration dependent N isotope fractionation.

Some studies were based on the relatively long life span of *F. vesiculosus,* and interpreted the isotopic composition of different sections of their thalli as the result of past pollution events (Savage and Elmgren 2004, Raimonet et al. 2013, Carballeira et al. 2014). The main assumption of these studies is that only the growing tips of the thallus take up nitrogen and, therefore, the isotopic composition of a given section of the thallus would reflect the isotopic composition of the dissolved nitrogen in the surrounding water at the time of growth. However, some questions need to be tested to fully interpret the data obtained in these studies. First, Fucaceae do not have a specific transport tissue, but the pores of the sieve plates should enable a continuous system of cytoplasm for the translocation of materials longitudinally (Moss 1983). There is experimental evidence of long distance transport of organic ^{14}C, ^{86}Rb or ^{32}P (Penot and Penot 1979, Diouris and Floc´h 1984, Raven 2003). If the transport of nitrogen along the thallus also exists, it would directly affect the retrospective identification of past nitrogen sources. Second, most studies assume that the isotopic composition of tissues does not change for at least several months, given that these species generally show low variability in $\delta^{15}N$ values at monthly time scales (Gartner et al. 2002, Raimonet et al. 2013) but no data of N-specific uptake and turnover rate were available for this species.

For assessing the feasibility of using *A. nodosum* and *F. vesiculosus* for the isotopic differentiation of local N sources, two sets of experiments were made under laboratory conditions. The first experiment aimed to determine the equilibration of N isotopes in the growing tips and older parts of the fronds by growing them under water with different N origins. The second experiment aimed to detect nitrogen transport along their thalli and to test if all the parts of the frond have the capacity of taking up NO_3^- by using artificially ^{15}N-enriched water. The latter approach also allowed the estimation of N turnover rates in different sections of the thallus.

Material and Methods

Experiment 1: N isotope equilibration

Water samples–The first laboratory experiment was conducted with water from 3 different sites: water from an urbanized watershed, from a forested watershed, and from an oceanic influenced site which was considered the control. The first two sites are Childs River (CR) and Sage Lot Pond (SLP), which are part of the Waquoit Bay National Estuarine Research Reserve, Massachusetts (Fig. 5.1). The Waquoit Bay estuarine system is a complex of sub-estuaries with different N inputs from their watersheds, and thus, with differing ambient N concentration and origin (Valiela et al. 1992, Valiela et al. 1997). The CR estuary (41°34'N, 70°32'W) is surrounded by the most urbanized watershed in the Waquoit Bay system. Nutrients (primarily nitrate) are delivered to the CR estuary from the watershed via groundwater flow (Valiela et al. 1992). In contrast, SLP (41°55'N, 70°50'W) is a forested watershed receiving a low N load, with NH_4^+ as the dominant dissolved inorganic nitrogen (DIN) form (Valiela et al. 1997) and is surrounded by salt marshes. The control site was placed at Nobska Beach (41°51'N, 70°65'W), which is an oceanic influenced site with no terrestrial or anthropogenic inputs draining in the area (Fig. 5.1a).

Experimental design– Individuals of *A. nodosum* and *F. vesiculosus* were collected at Quissett Harbor and Nobska Beach respectively, in Woods Hole, Massachusetts (Fig. 5.1a); and they were transported in coolers to laboratory. Macroalgae were kept in tanks with continuous seawater flow (15.7±1.6 °C) and low light intensities during the night (less than 12 hours) until the start of the experiment. *A. nodosum* individuals of 14.6±2.6 cm long and with 2 or 3 gas bladders, and *F. vesiculosus* individuals of 10.7±2 cm long were selected to run the experiment. Individuals with visible damage or epiphytes were avoided.

Macroalgae (n=4 for *A. nodosum*, n=3 for *F. vesiculosus*) were placed in three different 1 L Erlenmeyer flasks containing CR, SLP or Nobska unfiltered water. The study was run in triplicate with each replicate in a separate flask for each of the three treatments over a period of 22 days for *A. nodosum* and 12 days for *F. vesiculosus*. Samples were taken at the start of the experiment (t=0) and at subsequently exponential times. The different time scales for each species were chosen based on the previous knowledge of growth rates of the species. A control

flask with no macroalgae was established for each water treatment and maintained under the same conditions as the experimental flasks.

For comparison with experimental individuals, native individuals of *F. vesiculosus* were collected along with water samples where present (i.e. CR and SLP) and analyzed for stable isotope composition. Local populations of *A. nodosum* were not found at the sites selected for water collection.

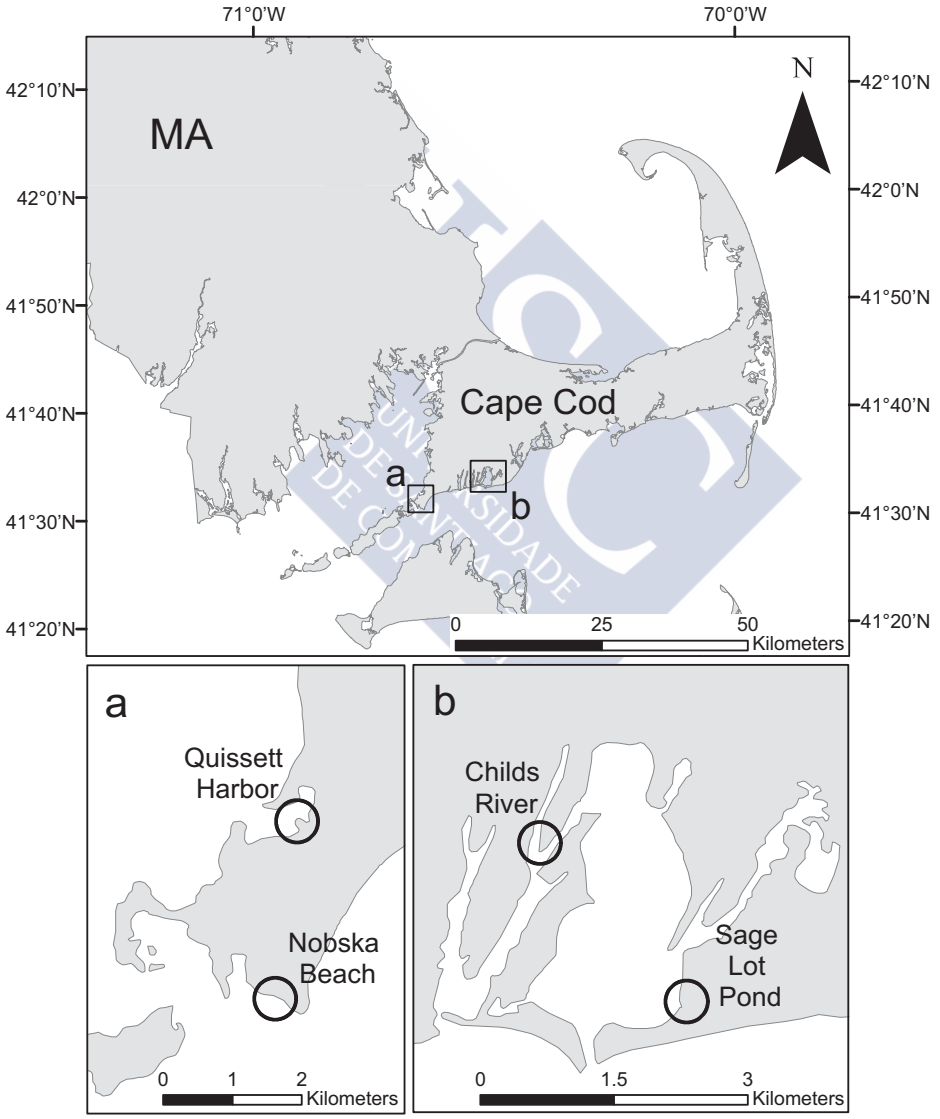

Figure 5.1. Location of the study sites at Cape Cod, Massachusetts, USA (Basemap: USGS).

Experiments were carried out in a culture chamber with 18:6 light:dark cycle at light intensities varying between 390-450 μE m^{-2} s^{-1} under 18-20 °C air temperature oscillation between night and day respectively. Water aeration was maintained with air pumps and diffusers and water temperature oscillated around 24.08±0.06 °C.

Water was replaced every 2 days to avoid nutrient depletion. Samples of water were collected before and after replacement to quantify the variation in DIN concentrations among times and sites and to check macroalgal consumption. Salinity and temperature were measured with a portable conductivity meter (YSI Model 30) every time the water was changed.

The macroalgal samples used for $\delta^{15}N$ and N and C content were separated with a glass spatula. The growing tip (1 cm) was sampled at all sampling dates during the experiment for both species. Additionally, at the start of the experiment (t=0) and at the endpoint, the growing tip (1 cm) and all intervesicular segments were sampled in *A. nodosum* individuals, while for *F. vesiculosus* individuals only the growing tip (1 cm) and the basal segment of the frond were sampled. All macroalgal samples were rinsed with Milli Q water and frozen (-20 °C) before processing. Later, samples were defrosted and dried (50 °C) until constant weight before grinding into a homogeneous powder prior to isotopic and elemental analysis.

Macroalgal growth–To measure macroalgal growth response to the different water samples, the wet biomass of each frond was recorded at the beginning of the experiment and at the time the frond was sampled. Individual growth rates (μ) were calculated as a percent increase in biomass per day (% d^{-1}):

$$\mu=100[\mathrm{Ln}(N_t/N_0)]/t$$

where N_t is the biomass on day t, N_0 is the initial biomass, and t is time in days of incubation (Lobban and Harrison 1994).

Nutrient sampling and analysis–Changes in concentration of NO_3^-+NO_2^-, NH_4^+, and PO_4^{3-} were determined during the experiment to quantify differences in ambient nutrient concentrations among water samples. Water samples were frozen until analysis of nutrient concentrations. Nitrate and phosphate were determined using standard colorimetric assays in a Lachat Auto Analyzer (Cd Reduction). Ammonium

concentrations were determined by spectrophotometry following the Indophenol method. Detection limit was 0.25 µM for any of the three nitrogen species.

Experiment 2: ^{15}N enrichment experiment

An enrichment experiment was done to determine N-turnover rates in different sections of the thallus and to test: i) the occurrence of transport of N along the thallus, from the tip to the basal segment of the frond, ii) the occurrence of transport of N from the basal segment of the frond to the tip, and iii) to quantify the uptake rates of the growing tips and mature parts of the thallus.

As in the previous experiment, *A. nodosum* and *F. vesiculosus* were collected at Quissett Harbor and Nobska Beach respectively (Fig. 5.1a). Macroalgae were transported in coolers to laboratory and maintained under the same pre-incubation conditions as previously described. For these experiments, *A. nodosum* individuals were 23.2±0.9 cm long and had 4 gas bladders, and *F. vesiculosus* individuals were 12.7±1.1 cm. The selected individuals did not show apparent damage or epiphytes. Treatment water was created by adding a stock solution of 10mM $K^{15}NO_3$ (99 atom % ^{15}N) to 2 L of a final volume of seawater. The final concentration was ~120 µM, with 98.8% atom % ^{15}N enrichment. Nitrate was selected as the tested nutrient as it is the dominant inorganic nitrogen compound in sewage.

To test i) and ii), experiments were divided in two periods: a first 4-h period under the stock solution, followed by a 24-h period under control seawater. During the first period, only the tips (i) or the basal segment of the frond (ii) of three different individuals of each species were submerged, while the non-submerged parts of the thallus were manually vaporized with control seawater at regular intervals (~20 min) to avoid desiccation. Macroalgae were maintained inside the culture chamber under the same light and temperature conditions as in the previous experiment. After this first 4-h period, individuals were gently washed with seawater and transferred individually to an Erlenmeyer flask with 1 L of control seawater. They were kept during 24 hours under the same conditions of temperature, light and aeration as in the previous experiment.

After both incubation periods, all individuals were immediately subsampled for stable isotope determinations. Each *A. nodosum* individual was divided into tip (1-1.5-cm fragment measured from the distal part) and intervesicular segments. Those of

F. vesiculosus were divided into tip (1-cm fragment from the distal part) and regular length segments (~3 cm) from the tip to the base. The lateral vegetative or reproductive branches of *A. nodosum* or reproductive tips of *F. vesiculosus* were discarded.

To test iii), the uptake capacity of the tip and non-growing parts of the thallus, three individuals of each species were completely submerged in the treatment solution for 2 h. Macroalgae were maintained inside the culture chamber under the light and temperature conditions as in the previous experiment. To exclude the possible transport of inorganic N along the thallus, macroalgae were subsampled immediately after the incubation period. Macroalgae were subsampled following the same procedure as previously described for i) and ii).

During each of the three treatments, control individuals of *A. nodosum* (n=3) and *F. vesiculosus* (n=3) were maintained in the same conditions as the experimental individuals but in 1L Erlenmeyer flasks with control seawater.

Internal nutrient content and $\delta^{15}N$ analysis

N stable isotope and elemental analyses for N and C content to estimate the tissue C:N were performed for all samples. Aliquots of ca. 2.5 mg of macroalgae samples were used. Samples were placed in tin capsules and introduced into an isotope-ratio mass spectrometer (Thermo Finnigan Mat Delta Plus) via an element analyzer (Carlo Erba CHNSO 1108). Isotopic results are expressed in delta notation:

$$\delta^{15}N = [(^{15}N_{sample}:^{14}N_{sample}/^{15}N_{std}:^{14}N_{std})-1] \times 1000$$

where the standard (std) is atmospheric N_2. Precision (se of 5 replicates) was better than 0.05‰ for either IAEA-N-2, IAEA-N-1 or IAEA-NO-3 standards. The coefficient of variation of triplicate sample aliquots was always <2%.

Statistical analyses and calculations

Comparison of nutrient concentrations among water samples was done by analysis of variance (ANOVA). This test was also used to analyze differences among sites and macroalgal segments along the thallus at the end of the isotope equilibration experiment, and to study differences between macroalgal segments within individuals from the same site. When significant differences were detected, *a posteriori* Student-Neuman-Keuls (SNK) tests for multiple comparisons were used to detect differences

among groups. Differences in the $\delta^{15}N$ and C:N in the growing tips of macroalgae over time were tested using a general linear model (GLM) univariate procedure using the site and time as fixed factors.

Experimental samples of the ^{15}N enrichment experiments were compared with the control samples to test the atom % ^{15}N enrichment using a paired-samples *t*-test, which compares two measurements of the same sample before and after the treatment. All tests were carried out with SPSS Statistical Software.

To estimate N uptake in the enrichment experiment we used the N specific uptake rate, which was calculated from appearance of the ^{15}N in the macroalgal tissue:

$$\text{N specific uptake} = (\text{atom\% } {}^{15}N_f - \text{atom\% } {}^{15}N_i)/R \cdot t$$

where atom % $^{15}N_f$ and atom % $^{15}N_i$ are the final and initial atom % ^{15}N enrichment of macroalgal thallus respectively, *R* (%) is the calculated exponential average of the initial and final atom % enrichment of NO_3^-, and *t* is the time in hours. The inverse of the N specific uptake-rate was used to estimate the turnover time (*tr*) in days that would take to renovate the total N of a particular macroalgal fragment.

Results

Experiment 1: N isotope equilibration rates

Concentrations of all inorganic nitrogen compounds during the experiment with *A. nodosum* in September were higher than those found during the *F. vesiculosus* experiment in August (Table 5.1). In the former case, water from CR had more nitrate and ammonium than water from the other sites but showed similar phosphate concentrations. In contrast, during the *F. vesiculosus* experiment, the oceanic-influenced site (Nobska) showed larger nitrate and lower ammonium and phosphate concentrations than those at the other experimental sites, which showed similar concentrations of all nutrients. In all cases, DIN:PO_4^{3-} values were low, indicating potential nitrogen limitation.

The macroalgal growth response to nutrient changes differed between species, although the pattern was very similar among sites within the same species (Fig. 5.2, Table 5.2). Overall the growth of *A. nodosum* was higher than the growth of *F. vesiculosus*.

In all cases there was positive growth at the end of the experiment, but maximum growth was recorded after 6 d for *A. nodosum* and after 12 d for *F. vesiculosus*. The increase in growth rates was almost continuous during the experiment with *F. vesiculosus* while growth slowed down between 6 and 12 d of the experiment in the case of *A. nodosum*. Nevertheless, growth rates of *A. nodosum* cultured under SLP water were higher than those measured under water from the other sites, while for *F. vesiculosus* maximum growth rates were observed for CR water (Table 5.2).

Table 5.1. Sampling dates, and mean (±se) values of salinity, nutrient concentrations (μM) and DIN:PO_4^{3-} ratio during the N isotope equilibration experiments with *A. nodosum* and *F. vesiculosus* exposed to water from Childs River, Sage Lot Pond and Nobska (Fig. 5.1). Significant differences among nutrient concentrations in the different sites are shown (*: P≤0.001, **: P≤0.01, ***: P≤0.05).

	A. nodosum			*F. vesiculosus*		
Variable	Childs River	Sage Lot Pond	Nobska	Childs River	Sage Lot Pond	Nobska
Dates	29 August- 20 September 2013			2 August- 14 August 2013		
Salinity	24.57±0.89	27.04±0.45	31.04±0.05	25.85±0.40	26.33±1.28	31.10±0.32
Nutrient concentrations (μM)						
$NO_3^- + NO_2^-$	5.98±2.58	2.08±0.29	1.85±0.14*	1.07±0.13	1.28±0.15	2.03±0.18**
NH_4^+	5.12±1.46	3.12±0.65	1.15±0.09**	2.19±0.01	0.85±0.13	0.57±0.04***
PO_4^{3-}	1.70±0.51	1.06±0.12	1.25±0.12	1.55±0.24	0.75±0.15	1.23±0.09**
DIN:PO_4^{3-}	7.02±2.38	4.99±0.79	2.20±0.3	1.11±0.33	2.29±0.78	2.39±0.55*

Table 5.2. Results of the general linear model (GLM) univariate procedure to analyze the variability in growth (% d^{-1}), $\delta^{15}N$ (‰) or C:N in *A. nodosum* and *F. vesiculosus* when grouped by sites (Childs River, Sage Lot Pond or Nobska) and sampling times, as fixed factors.

		Growth					$\delta^{15}N$					C:N				
Species	Factor	SS	df	MS	F	P	SS	df	MS	F	P	SS	df	MS	F	P
A. nodosum																
	Intercept	91.6	1	91.6	102.2	<0.001	362.8	1	362.8	2282.2	<0.001	7645.8	1	7645.8	77.6	<0.001
	Site	0.4	2	0.2	0.2	0.82	0.6	2	0.3	2.0	0.147	351.7	2	175.8	1.8	.181
	Time	75.8	4	18.9	21.1	<0.001	3.4	1	3.4	21.4	<0.001	493.5	1	493.5	5.0	.031
	Site x Time	70.9	8	8.9	9.9	<0.001	0.6	2	0.3	2.0	0.145	310.1	2	155.0	1.6	.220
	Error	26.9	30	0.9			6.2	39	0.2			3840.9	39	98.5		
	Total	265.5	45				2370.7	45				68363.6	45			
F. vesiculosus																
	Intercept	0.3	1	0.3	4.4	0.04	436.9	1	436.9	3031.0	<0.001	3168.8	1	3168.8	145.7	<0.001
	Site	0.1	2	0.0	0.6	0.57	0.3	2	0.2	1.1	0.349	26.6	2	13.3	0.6	0.55
	Time	4.5	1	4.5	65.8	<0.001	0.2	1	0.2	1.7	0.198	531.0	1	531.0	24.4	<0.001
	Site x Time	0.4	2	0.2	2.8	0.08	0.8	2	0.4	2.9	0.068	145.7	2	72.9	3.3	0.049
	Error	2.0	30	0.1			4.3	30	0.1			652.5	30	21.7		
	Total	9.2	36				2742.9	36				37431.6	36			

The response of N isotope composition was different for each species (Fig. 5.2) but similar for all water types assayed (Table 5.2). While for *A. nodosum*, $\delta^{15}N$ values in the growing tips significantly increased with time rapidly exceeding the range of initial values, those for *F. vesiculosus* did not vary as a factor of time or site and their range of variation was in most cases within the range of variation of the initial values (6.7±0.1‰ in *A. nodosum* and 8.5±0.2‰ in *F. vesiculosus*, Fig. 5.2). These changes were not large enough to reach the N isotopic values observed in native individuals of *F. vesiculosus* in CR (6.9±0.1‰) or SLP (5.0±0.3‰).

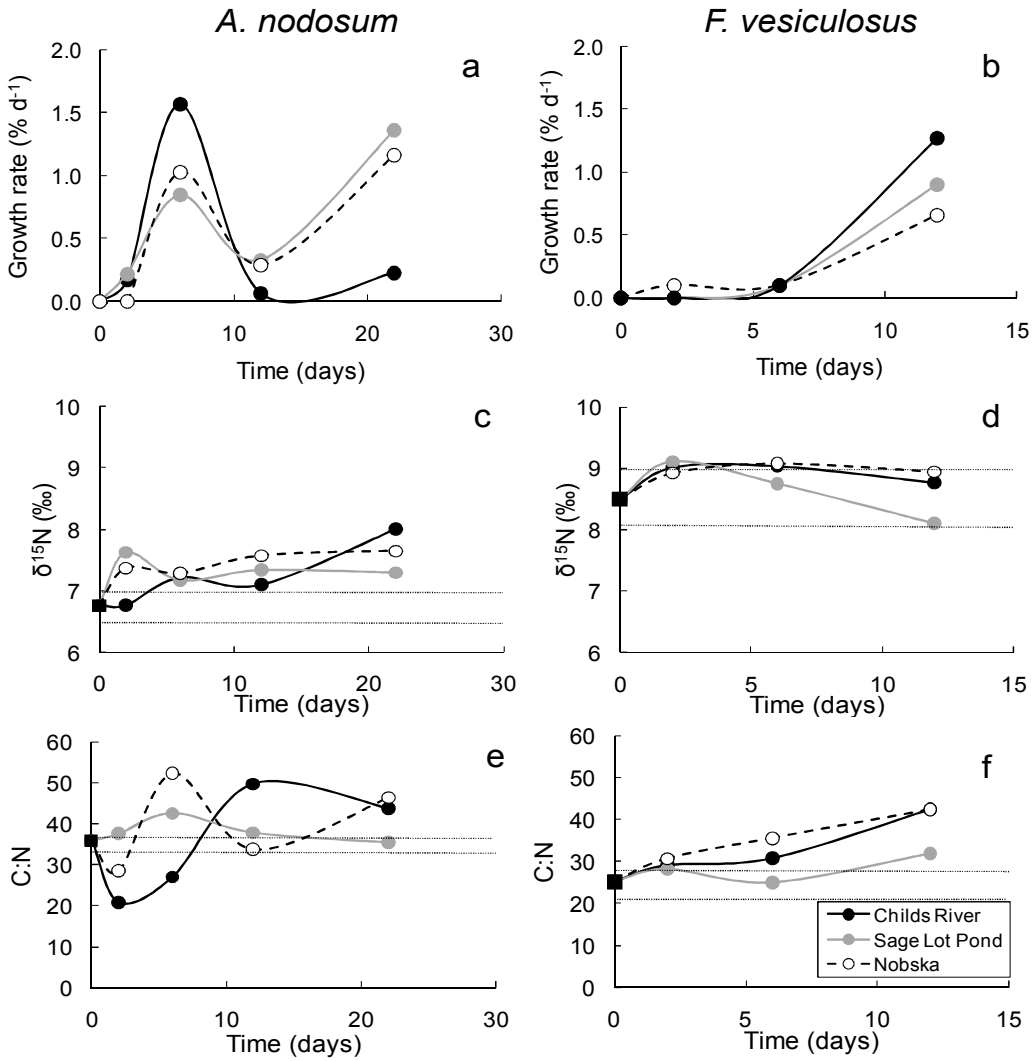

Figure 5.2. Changes in mean (n=3) growth in wet biomass (% d^{-1}), $\delta^{15}N$ (‰) and tissue C:N in *A. nodosum* (a, c, e) and *F. vesiculosus* (b, d, f) during 22 and 12 d incubations respectively using water of three different locations. Square symbols are the mean values at time 0 and the dashed lines their range of variation. Analysis of variance results are shown in Table 5.2.

As observed in the case of growth rates, tissue C:N of both species increased during the experiment but in this case there was no significant effect of the culturing water employed and only *F. vesiculosus* maintained in SLP water had lower C:N values than those individuals maintained in other water types (Fig. 5.2, Table 5.2). For all treatments, however, the final C:N values measured exceeded the range of values observed in the site of collection.

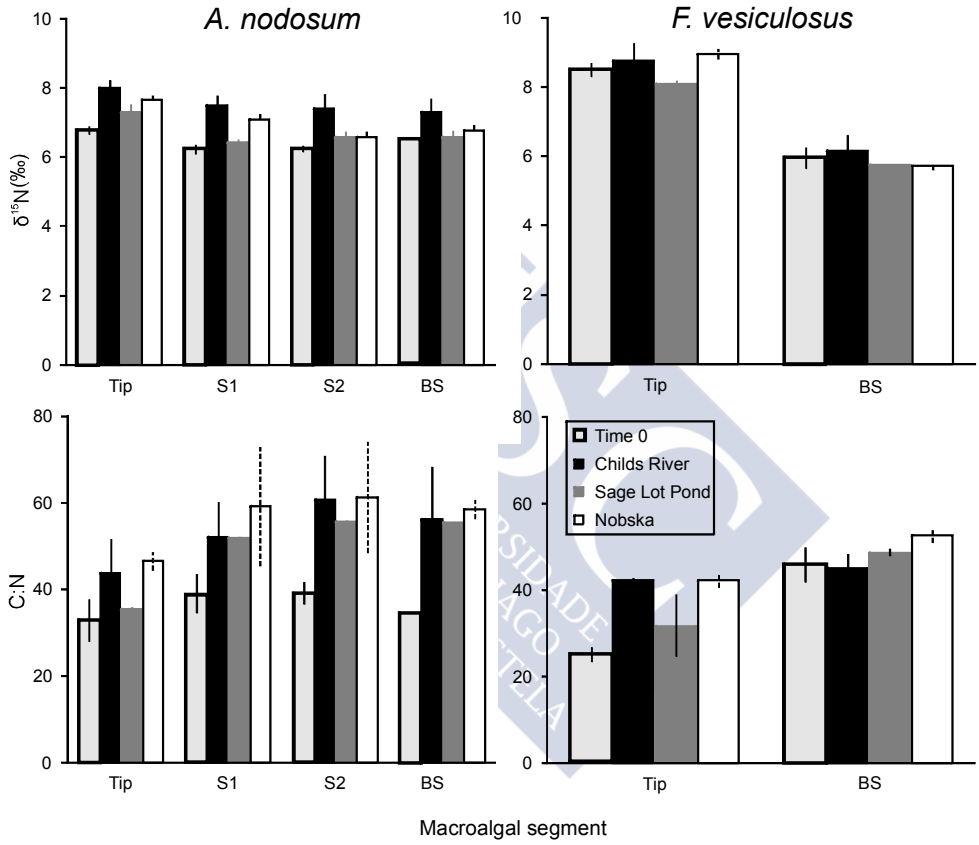

Figure 5.3. Initial (Time 0) and endpoint variation of $\delta^{15}N$ values (mean±se, ‰) and tissue C:N (mean±se) for different sections of the thallus of *A. nodosum* (left panels) and *F. vesiculosus* (right panels) individuals (n=3) growing under water of three different locations (Childs River, Sage Lot Pond and Nobska). The Tip and BS segments correspond to the growing apical segment and the basal segment respectively. S1 and S2 segments for *A. nodosum* correspond to the intervesicular segments numbered from the tip to the base.

At the end of the experiment, differences between initial and final values along the thallus were especially noticeable in the tips, both for $\delta^{15}N$ and tissue C:N values (Fig. 5.3, Table 5.3). In all parts of the frond, and for both species, the lowest isotopic

values were observed generally in individuals cultured in SLP water and the highest values in those cultured in CR water (Fig. 5.3) thus approaching the isotopic values of native macroalgae. The $\delta^{15}N$ values for growing tips of *A. nodosum* individuals maintained in Nobska and SLP water were significantly different from other segments, while no significant differences between segments from the same individual exposed to CR water appeared (ANOVA, post hoc SNK test, P≤0.01). *F. vesiculosus* showed significant differences between tip and the basal segment of the frond in individuals under all culture regimes (ANOVA, post hoc SNK test, P≤0.01).

Table 5.3. Results of the analysis of variance (one-way ANOVA) and SNK post-hoc comparison tests of $\delta^{15}N$ (‰) and C:N in the different macroalgal segments of *A. nodosum* and *F. vesiculosus* individuals (n=3) at the endpoint of the study, compared with the initial values of macroalgae (t0) (Fig. 5.3). Sites were set as the fixed factors: CR, Childs River; SLP, Sage Lot Pond and N, Nobska. n.s.: non significant. BS: basal segment of the frond.

		$\delta^{15}N$		C:N	
Species	Macroalgal segment	P value	post-hoc	P value	post-hoc
A. nodosum					
	Tip	0.000	t0<SLP<CR=N	0.066	n.s.
	S1	0.002	t0<SLP<CR=N	0.428	n.s.
	S2	0.007	CR>t0=SLP=N	0.340	n.s.
	BS	0.001	CR>SLP=N>t0	0.007	t0<CR=SLP=N
F. vesiculosus					
	Tip	0.016	t0=SLP<CR=N	0.012	t0=SLP<CR=N
	BS	0.625	n.s.	0.238	n.s.

Experiment 2: ^{15}N enrichment experiment

The growing tip and the basal segment of the frond of both species when submerged in ^{15}N enriched seawater significantly increased their ^{15}N content relative to non-submerged parts of the frond and to control segments (Fig. 5.4a, b). Tips increased from natural levels to average enrichments of 1.1% and 1.7% in *A. nodosum* and *F. vesiculosus* respectively, while enrichment of the basal segment was only 0.44 and 0.76%, respectively. No evidence of enrichment was found in the emerged sections of the thallus during this experiment.

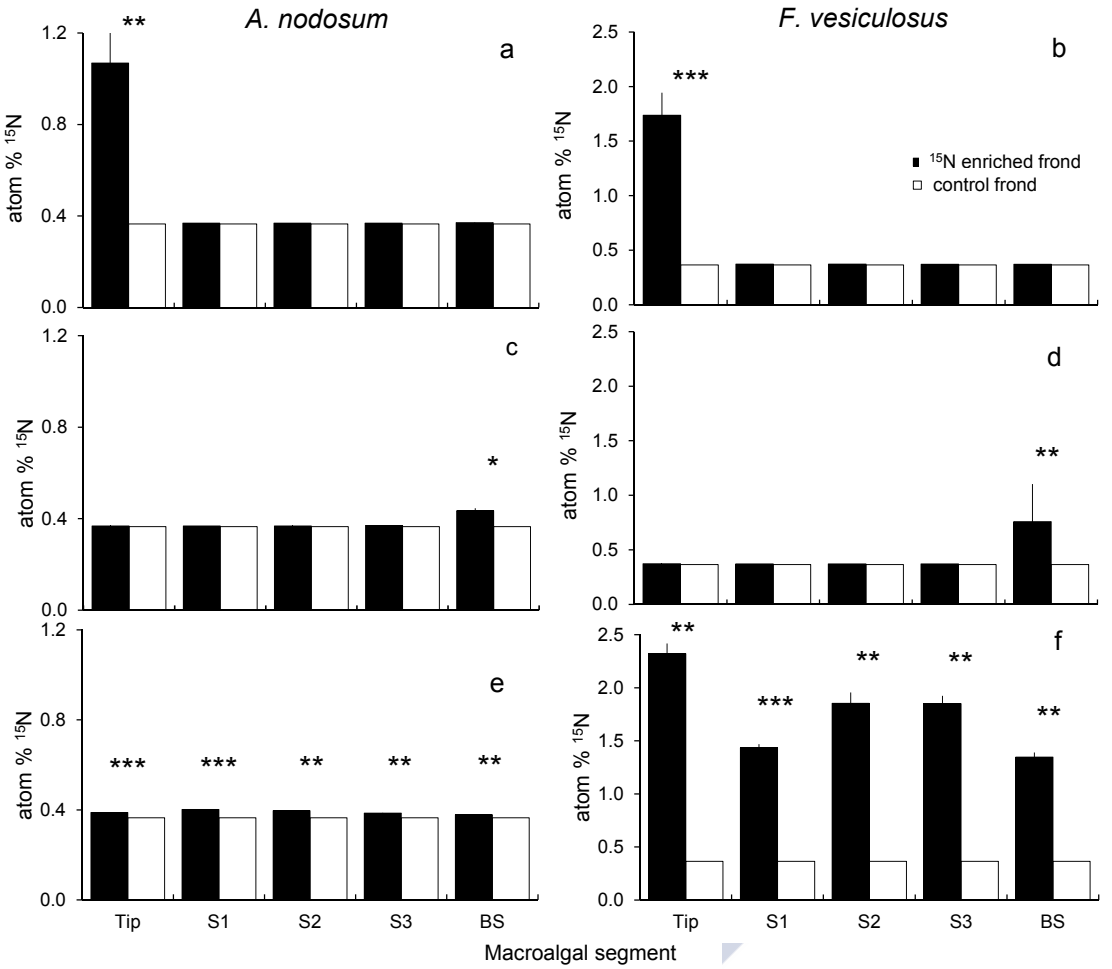

Figure 5.4. Mean (±se) variation of atom % ^{15}N enrichment along the fronds of *A. nodosum* (a, c, e) and *F. vesiculosus* (b, d, f) individuals (n=3) when either: the tip was submerged in an enriched seawater solution (a, b), the basal segment of the frond (BS) was submerged in an enriched seawater solution (c, d), or the entire frond was submerged in an enriched seawater solution (e, f). The tip in both species corresponds to the growing apical segment. S1, S2 and S3 segments correspond to the intervesicular segments and to 3-cm segments in order to the closeness to the tip in *A. nodosum* and *F. vesiculosus* respectively. Significant differences between the experimental and the control frond values are indicated by asterisks (*: P≤0.05, **: P≤0.01, ***: P≤0.001, paired-samples *t*-test). Notice the different scales of the y-axis for both species.

The ^{15}N content in wholly-submerged fronds of both species significantly changed after the treatment (Fig. 5.4c). As in the previous experiment, higher enrichment was observed for *F. vesiculosus* than for *A. nodosum* individuals, and consequently N-specific uptake rates were lowest in the latter (Table 5.4). Among *A. nodosum* individuals, the basal segment showed the lowest enrichment, while in *F. vesiculosus*

the segment immediately under the growing tip showed the lowest enrichment together with the basal segment. The tips of both species were more enriched relative to other segments.

Table 5.4. Variation of mean±se N specific uptake (d^{-1}) and turnover time (d) in the different macroalgal segments of *A. nodosum* and *F. vesiculosus* when, i) the tip was submerged in an enriched seawater solution (Fig. 5.4a,b), ii) the basal segment of the frond (BS) was submerged in an enriched seawater solution (Fig. 5.4c,d), and iii) the entire frond was submerged in an enriched seawater solution (Fig. 5.4e,f). The tip in both species correspond to the growing apical segment. S1, S2 and S3 segments correspond to the intervesicular segments and 3-cm segments in order to the closeness to the tip in *A. nodosum* and *F. vesiculosus* respectively.

Species	Experiment	Macroalgal segment	N specific uptake	Turnover time
A. nodosum				
	i	Tip	0.0409±0.0104	29.27±9.62
	ii	BS	0.0048±0.0004	209.17±14.66
	iii	Tip	0.0053±0.0001	188.15±5.33
		S1	0.0085±0.0002	118.04±2.44
		S2	0.0087±0.0007	115.97±10.27
		S3	0.0062±0.0004	162.43±10.8
		BS	0.0044±0.0001	227.67±5.57
F. vesiculosus				
	i	Tip	0.0665±0.0100	15.74±2.35
	ii	BS	0.0525±0.0100	19.06±2.35
	iii	Tip	0.0949±0.0047	10.59±0.5
		S1	0.0522±0.0017	19.2±0.62
		S2	0.0722±0.0050	13.98±0.9
		S3	0.0721±0.0036	13.95±0.73
		BS	0.0476±0.0021	21.1±0.96

However, N uptake proceeded at low rates and N turnover times estimated from these rates were in general higher than the duration of the isotope equilibration experiments (Fig. 5.2). The average N turnover time of tip-submerged individuals was ca. 30 and 16 d for *A. nodosum* and *F. vesiculosus* respectively (Table 5.4). In contrast, when the basal segment was submerged, individuals showed N turnover times that averaged 7 months and 19 days for *A. nodosum* and *F. vesiculosus*, respectively.

Finally, when all the frond segments were submerged the turnover time of the tip for *A. nodosum* was longer (up to 6 months) than in the previous treatments, although the turnover at the basal segment of the frond was maintained (Table 5.4). Turnover time for intermediate segments was slightly faster (4-5 months) than at the tip or at the basal segment. In the case of *F. vesiculosus*, N turnover at the tip would need on average 11 d and only 21 d at the basal segment of the frond, while other algal segments showed intermediate turnover values.

Discussion

Variation of $\delta^{15}N$ in macroalgal growing tips

As both macroalgae show apical growth, isotope composition of the tips is expected to change according to the isotope composition of the surrounding water at faster rates than other parts of the thallus. These changes would ideally lead to a complete isotope equilibration between the algal tissue and the water in absence of isotope fractionation. The results of the experiments in this study revealed that the tips of both *A. nodosum* and *F. vesiculosus* required long time to converge with the $\delta^{15}N$ values typical of native plants when exposed to water with different isotopic composition. The time required largely exceeded the duration of the experiments (up to 22 d), as N turnover rates varied between 11 d (*F. vesiculosus*) and 6 months (*A. nodosum*). Similar delays in the equilibration of $\delta^{15}N$ values in apical tissues of *F. vesiculosus* when changing the surrounding water were reported in other studies (Deutsch and Voss 2006) while much faster equilibration was observed for other brown (García-Sanz 2009), red or green macroalgal species (Naldi and Wheeler 2002, Teichberg et al. 2008). Such delays can be due to slow growth and N uptake rates, strong isotope fractionation, low ambient N or to the initial nitrogen content and isotope composition of the individuals assayed.

Both macroalgae have logistic growth, with the highest rates during their first year of life. *F. vesiculosus* can grow in length up to 2 cm mo^{-1} at the season of maximum growth, but more often rates are as low as 0.6 cm mo^{-1} (Viana et al. in review b). The growth for *A. nodosum* is much slower, but individuals of this species can live for more than 10 yr (Viana et al. in press). Low growth rates also imply lower N requirements and uptake than fast growing species (Pedersen and Borum 1997). Such low requirements would explain N-specific uptake rates <0.1 d^{-1} even at high ambient N concentrations as those employed in the enrichment experiment in this

study (Table 5.4), and consequently long N turnover times in these macroalgae.

Strong isotope fractionation during uptake is not likely to occur. Previous studies with Fucaceae (García-Sanz 2009) and other macroalgae (Cohen and Fong 2005) did not find significant N isotope fractionation related to nutrient concentrations, in contrast with diatoms (Wada and Hattori 1978, Pennock et al. 1996). The rates of change in $\delta^{15}N$ in our experiments would have been faster than observed if fractionation were a significant factor, as the light isotopes would have been preferred. For instance, the assayed *F. vesiculosus* with mean initial $\delta^{15}N$=8.5‰ would have converged to values typical of individuals native of the water origin locations (5.0 to 6.9‰) but they did not show significant changes in their isotopic composition after 12 d.

The concentration of ambient N may have also affected changes in macroalgal $\delta^{15}N$. The water employed in the experiments had nutrient concentrations typical of summer in the study area, when uptake by primary producers depletes nutrients (Tomasky et al. 1999). However, N sources, rather than total N concentration determines $\delta^{15}N$ in the water and ultimately in primary and secondary producers (McClelland and Valiela 1998b, Viana and Bode 2013). Experiments with other species showed that macroalgal $\delta^{15}N$ did not change with water N concentrations as long as the $\delta^{15}N$ of dissolved N was constant (Cohen and Fong 2005, García-Sanz 2009). Furthermore, nutrient uptake in *F. vesiculosus* is less dependent on substrate concentration than in green or red algae (Pedersen and Borum 1997). In our experiment with water of different origins, the low concentrations of dissolved N did not prevent the individuals of both species from growing in weight and maintaining C:N values characteristic of non N-limited algae (Niell 1976), thus suggesting that the slight changes in $\delta^{15}N$ were not a direct consequence of water N concentration.

The relatively high nitrogen content (1.2±0.3% for *A. nodosum*, 1.4±0.1% for *F. vesiculosus*) and the enriched $\delta^{15}N$ values of macroalgae at the starting point could have also influenced isotopic equilibration. Slow-growing brown macroalgae usually rely on their internal N pools during periods of low nutrient supply, as in summer seasons in temperate areas (Lehvo et al. 2001, Villares et al. 2013). During these periods growth rates and external nutrient demand are lowered while the macroalgae, eventually profiting from high light levels, develop carbon reserves, thus increasing tissue C:N, as observed in our experiments (Fig. 5.2). Naldi and Wheeler (2002) also observed that high total N content of thalli influenced nitrate uptake rates in green

and red macroalgal species. Low external N demand along with large difference in $\delta^{15}N$ values between the macroalgal tissue and the surrounding water (as suggested by the $\delta^{15}N$ values of native macroalgae), may be the main determinants of the rate of isotopic equilibration in our incubations with *F. vesiculosus*. Other experiments with transplanted individuals of this species in the field also found small or no changes in their tissue $\delta^{15}N$ after days of incubation (Deutsch and Voss 2006). In contrast, and despite the longer turnover time, *A. nodosum* started to show differences in $\delta^{15}N$ after 12 days of incubation, likely because the initial values for this species were much lower than those for *F. vesiculosus*.

N uptake and turnover along the thallus

The results of the enrichment experiments showed that both species do not transport recently absorbed N along their thallus, at least during 24 h after uptake (Fig. 5.4). Despite their internal structure (i.e. symplastic pathway) suited for transport (Raven 2003), only carbon photosynthetic assimilates were reported to translocate along the thallus of some Fucaceae (Diouris and Floc´h 1984). Inorganic nitrogen transport, however, was reported for other brown macroalgae, such as Laminariales (Mizuta et al. 1996, Hepburn et al. 2012). These algae have nutrient requirements different from those of Fucales as they show basal meristematic growth, which means that they grow where the blade and the stipe meet (Lobban and Harrison 1994). In contrast, Fucales show mostly apical growth and therefore concentrate N demands in the tips of the thallus (Topinka Bigelow 1978), although as demonstrated by our enrichment experiment (Fig. 5.4c), all sections of the thallus are able to take up inorganic N from the water. As N transport have relatively high energy and oxygen requirements (Raven 2003), this process can be avoided if both assimilation and uptake occur in the same part of the thallus. In Laminariales, N uptake and assimilation occur at different rates in the different parts of the thallus, deriving in gradients along the frond (Mizuta et al. 1996).

Despite their apical growth, variation in $\delta^{15}N$ values along the thallus has been reported for *Fucus* species (Savage and Elmgren 2004, Raimonet et al. 2013) and in the present study (Fig. 5.3). If transport is excluded, such intraindividual variation might be due to differential uptake and growth, or to isotope fractionation in the different sections of the thallus.

In the enrichment experiment we showed that both species were able to incorporate dissolved nitrogen when submerged (Fig. 5.4). The process of nitrogen uptake and assimilation in macroalgae involves transport from the water column and then assimilation into organic compounds, followed by incorporation into proteins and macromolecules for growth (McGlathery et al. 1996). Growth is the most important N sink in macroalgae. In mature segments, N demand for structural pools is not as important as in growing tips, this would explain why N uptake at the non-growing segments was only half the uptake rate measured at the tips of *F. vesiculosus* when all the frond was submerged (Table 5.4). For *A. nodosum* there was also a marked difference in the uptake rates of the tip and those of the mature segments, at least when only one of the sections was submerged. These results agree with studies reporting higher N uptake in apical fronds and whole young plants or germlings and lowest in slower-growing older fronds and stipes of *F. spiralis* (Topinka Bigelow 1978, Rosenberg et al. 1984) and differential ^{15}N enrichment along thalli regions of *F. vesiculosus* (Döhler et al. 1995).

Non-apical segments of *A. nodosum* and *F. vesiculosus* individuals can store N to use in metabolic processes other than growth. For instance, N can be accumulated as inorganic (NO_3^- and NH_4^+) and organic compounds (as phycobiliproteins) and can be found in algal pigments (Hanisak 1983) although NH_4^+ storage capacity is limited due to toxicity (Haines and Wheeler 1978, Lotze and Schramm 2000).

The net short-term N uptake recorded along the thallus implies that $\delta^{15}N$ values of different sections would change with the isotopic composition of the surrounding water at rates depending on their initial $\delta^{15}N$ value, and of the processes affecting isotope fractionation within each section. Nitrogen release, both in organic and inorganic forms, has been observed for some green and red macroalgae (Naldi and Wheeler 2002, Tyler and McGlathery 2006) and was interpreted as the result of isotopic equilibration of internal and external pools (Fujita et al. 1988) or to stress due to sudden changes in the proportion of different N sources (Naldi and Wheeler 2002). Even when fractionation during uptake, resulting in tissue $\delta^{15}N$ values lower than those of the water, is not likely (García-Sanz 2009), the release of preferentially light N isotopes may explain the higher enrichment of the tip sections compared to other parts of the thallus, as found in our experiments (Figs. 5.3 and 5.4) and in other studies (Raimonet et al. 2013). As far as we know, there are no reports of N release in the species considered in our study, but it can be expected that this process is restricted to the most metabolically active tissues.

Implications for the use of* A. nodosum *and* F. vesiculosus *to monitor land-derived nitrogen sources

The results of the present study are of application when using *A. nodosum* and *F. vesiculosus* to study the impact of anthropogenic N sources on littoral ecosystems both analyzing native populations and in incubation experiments, the latter applicable when these species are not naturally present in the impacted area. Taking advantage of the apical growth and long life span of both species, Savage and Elmgren (2004) interpreted $\delta^{15}N$ values in different sections of the thallus of *F. vesiculosus* in a retrospective study to monitor changing N loadings. The underlying assumptions were that annual growth occurred only at the tips and, by knowing the rate of growth, each section of the thallus could be dated and associated to a particular period of exposure to the ambient N. Thus, $\delta^{15}N$ of the sections would reflect past N sources if mature segments do not equilibrate N contents with the surrounding water and if there is no transport of N along the thallus. Other studies, however, questioned this application for retrospective studies as they found contrasting patterns of change along the thallus that could not be related to ambient N (Raimonet et al. 2013, Carballeira et al. 2014).

The enrichment experiment in this study demonstrated that all sections of the thallus of both species take up N from the ambient water when submerged. Even when there was no transport of the N along the thallus and the rates of uptake at the mature parts of the frond were lower that at sections located at or near the tip this uptake would affect the $\delta^{15}N$ of the sections. These results explain why previous studies found contrasting patterns of change of $\delta^{15}N$ along the thallus of *F. vesiculosus* (Carballeira et al. 2014) as the $\delta^{15}N$ of each section changes with the isotopic composition of the water at different rates. Therefore, it is not possible to obtain unbiased estimates of past N sources from the $\delta^{15}N$ of different sections of the thallus of these macroalgae. Furthermore, determinations of $\delta^{15}N$ from pooled samples of different sections would produce $\delta^{15}N$ values resulting from a mixture of past and present N sources, depending on the amount of matter from sections with different turnover rates. Pooled samples of the whole individual can be also be misinterpreted if individuals of different lengths (i.e. ages) are used. However, $\delta^{15}N$ of the tips can be used as monitors of N sources in the ambient water averaged over scales of 15 days (*F. vesiculosus*) and up to 6 months (*A. nodosum*). This range of integration times is particularly appropriate to differentiate chronic pollution from point discharges that may have little impact on the macroalgae.

Besides the use of natural populations, these macroalgae can be used in transplantation or laboratory experimental incubations with different water types to determine potential impacts of different N sources (Deutsch and Voss 2006). In this case, the turnover and equilibration times of the tips, as determined in the present study, need to be taken into account when determining the duration of the incubations. Otherwise the results will not reflect the actual impact of the ambient N sources.

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*Variability in $\delta^{15}N$ of intertidal brown algae along a salinity gradient: differential impact of nitrogen sources**

Abstract

While it is generally agreed that $\delta^{15}N$ of brown macroalgae can discriminate between anthropogenic and natural sources of nitrogen, this study provides new insights on net fractionation processes occurring in some of these species. The contribution of continental and marine sources of nitrogen to benthic macroalgae in the estuary-ria system of A Coruña (NW Spain) was investigated by analyzing the temporal (at a monthly and annual basis) and spatial (up to 10 km) variability of $\delta^{15}N$ in the macroalgae *Ascophyllum nodosum* and three species of the genus *Fucus* (*F. serratus*, *F. spiralis* and *F. vesiculosus*). Total nitrate and ammonium concentrations and $\delta^{15}N$-DIN, along with salinity and temperature in seawater were also studied to address the sources of such variability. Macroalgal $\delta^{15}N$ and nutrient concentrations decreased from estuarine to marine waters, suggesting larger dominance of anthropogenic nitrogen sources in the estuary. However, $\delta^{15}N$ values of macroalgae were generally higher than those of ambient nitrogen at all temporal and spatial scales considered. This suggests that the isotopic composition of these macroalgae is strongly affected by fractionation during uptake, assimilation or release of nitrogen. Besides, the absence of correlation between macroalgal and water samples, suggests that the $\delta^{15}N$ of the species considered can be used to determine the impact of the different nitrogen sources integrated over long-time periods, but not for monitoring short-term changes.

KEYWORDS:

Fucus
Ascophyllum
monitoring
DIN
coast
salinity gradient

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Introduction

Coastal areas integrate the nitrogen inputs from continental and marine sources. The growing anthropogenic pressure in these areas has increased dissolved inorganic nitrogen (DIN) concentrations in continental sources in comparison with seawater. Nitrogen concentrations could also be affected by other nitrogen inputs, as atmospheric deposition and upwelled seawater, and by processes as nitrogen uptake by primary producers, regeneration and movement of dissolved organic nitrogen (DON) at the sediment surface (Fry 2002). Therefore, monitoring nutrient concentrations is not enough to identify the origin of DIN in coastal areas (Sebilo et al. 2006). To overcome this constrain, the ratio of nitrogen stable isotopes ($\delta^{15}N$) has been increasingly used as marker of anthropogenic nutrient loading in the last years (McClelland and Valiela 1998b, Ahad et al. 2006, Deutsch and Voss 2006, Schubert et al. 2013).

The direct measurement of stable isotopes on DIN has been widely used and characteristic isotopic signatures were identified for different nitrogen origins (Heaton 1986, Ahad et al. 2006, Raimonet et al. 2013, Viana and Bode 2013). Human and animal wastewaters are enriched in ^{15}N relative to seawater because of strong isotopic fractionation during nitrification and volatilization in the case of NH_4^+, or denitrification in the case of NO_3^- (Mariotti et al. 1981). In contrast, synthetic fertilizers are depleted in ^{15}N due to the atmospheric origin of the fixed nitrogen (Heaton 1986). Consequently, $\delta^{15}N$ values of marine organisms show large variability in human impacted sites (McClelland and Valiela 1998b, Fry et al. 2003, Bode et al. 2014).

Macroalgae have been traditionally employed as biomonitors of eutrophication because their growth is rapidly enhanced by nutrient inputs (McClelland and Valiela 1998b, Piñón-Gimate et al. 2009). The $\delta^{15}N$ of the different macroalgal species shows a large variation, from 0.2 to 50.1‰ (Dailer et al. 2010). Besides the generalized use of macroalgae for monitoring nitrogen sources, only recent studies have addressed the influence of intrinsic or external factors on the variability of the isotopic values. The main intrinsic factors affecting variability of macroalgal $\delta^{15}N$ are the preferential mobilization of light isotopes (isotopic fractionation) during uptake, excretion and metabolic reactions (Teichberg et al. 2008) and intra-frond variability (Savage and Elmgren 2004, Raimonet et al. 2013, Carballeira et al. 2014, Viana et al. in review a). External factors are littoral position (Kim et al. 2013) or light (Dudley et al. 2010).

Fucoid species are long-lived species appropriate for detecting chronic nitrogen loadings and to obtain long-time integrative measures (Savage and Elmgren 2004, Bode et al. 2011b, Carballeira et al. 2013, Viana and Bode 2013). As they are perennial, and they live in the intertidal area of a wide variety of environments; they are subject to a wide spatial and seasonal variability of abiotic factors and nitrogen sources. This variability might significantly influence the $\delta^{15}N$ in their tissues, but it is not known until what extent the variability observed in some studies is due to the sources or to the nitrogen metabolism in macroalgae (i.e. net fractionation of all processes occurring within macroalgal tissues). Simultaneous measures of both macroalgal and water $\delta^{15}N$ are thus required to detect the sources of this variability (Deutsch and Voss 2006, Raimonet et al. 2013, Viana and Bode 2013).

The coast of Galicia (NW Spain) is characterized by the presence of numerous estuaries and rias (tidal inlets) that are dually influenced by oceanic and terrestrial inputs (Viana and Bode 2013). Oceanic inputs are characterized by spring-summer upwelling processes. This phenomenon naturally fertilizes the surface water from rias, and the initial inputs are amplified by remineralization of organic matter in the shelf and subsequent import with estuarine circulation (Álvarez-Salgado et al. 1996). Terrestrial inputs are represented by independent river basins draining in each ria. Riverine nutrient concentrations are generally higher than oceanic waters, but the low river flows compared to the water volume exchange by tides derives in a small effect of the river water in the rias (Álvarez-Salgado et al. 1996, Bode et al. 2011b). Nutrient concentrations of these continental inputs may be increased by the presence of urban nuclei, and anthropogenic activities, as agriculture or cattle breeding. This region is then a proper scenario for studying the variability of stable isotopes due to the different nutrient sources present in the area (Bode et al. 2006, 2011b, 2014; Carballeira et al. 2013, Viana and Bode 2013). The variability of the nitrogen content of any effluent entering coastal waters is of considerable importance to management since it controls the level of primary production (Valiela 1995), and it may produce an imbalance between denitrification processes and inputs.

As brown macroalgae can discriminate between anthropogenic and natural nitrogen sources, variable N isotopic values were observed along impact sites (Gartner et al. 2002, Carballeira et al. 2013). The aim of this study is to determine if this variability of isotopic values is due to the differential impact of nitrogen sources or also to local or intrinsic factors of macroalgae. The impact of different

nitrogen sources on the $\delta^{15}N$ of intertidal Fucaceae, *A. nodosum* and the genus *Fucus* (*F. serratus*, *F. spiralis* and *F. vesiculosus*) along a salinity gradient was studied for the application of these species as biomonitors of nitrogen sources. As they are long-living species and show slow growth rates, long temporal scales (monthly and annual basis) were taken into account. To assess the possible origin of the isotopic variability in macroalgae, $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ were also determined, along with nutrient concentrations, temperature and salinity of the seawater.

Material and Methods

Study site

The study was conducted at Ría de A Coruña, NW Spain (Fig. 6.1), which can be divided in two areas. The inner part of the ria, called Ría do Burgo, has a steep salinity gradient and estuarine characteristics, with a mean depth of 10 m. The outer bay is 6 km long and has a width in the mouth of about 3 km, constituting a total area of about 24 km^2 (Varela et al. 1994). The bay includes a harbor area and a seawall, and it has a high influence of marine waters (Cabanas et al. 1987). The river Mero drains in the area, but the inorganic nutrient inputs of the river are considered to be less important than the fertilization by the upwelling regimes, especially in the bay. Dense population nuclei can be found at both sides of the ria, especially around the estuarine zone and in the western part (left bank) of the bay, the latter occupied by the city of A Coruña (~240,000 inhabitants). In contrast, the northern and eastern margins of the bay are characterized by mostly rural and residential landscapes. Several fucoid species are well distributed in the rocky intertidal areas of this ria from semi-exposed to wave protected areas (Bárbara et al. 1995). Only in the inner and middle estuary, sedimentation and accumulation of mud is noticiable, and other species as vascular plants (as *Juncus* sp. or *Spartina* sp.) or *Zostera noltii* are present (Bárbara et al. 1995).

Sampling

To determine the impact of the different nitrogen sources on macroalgal and water nitrogen isotopic values and on nutrient concentrations, sampling was carried out at various spatial and seasonal scales. Fucoid species were selected and sampled according to their availability along the ria: *Ascophyllum nodosum*, and one of several species of the genus *Fucus* (*F. serratus*, *F. spiralis* and *F. vesiculosus*).

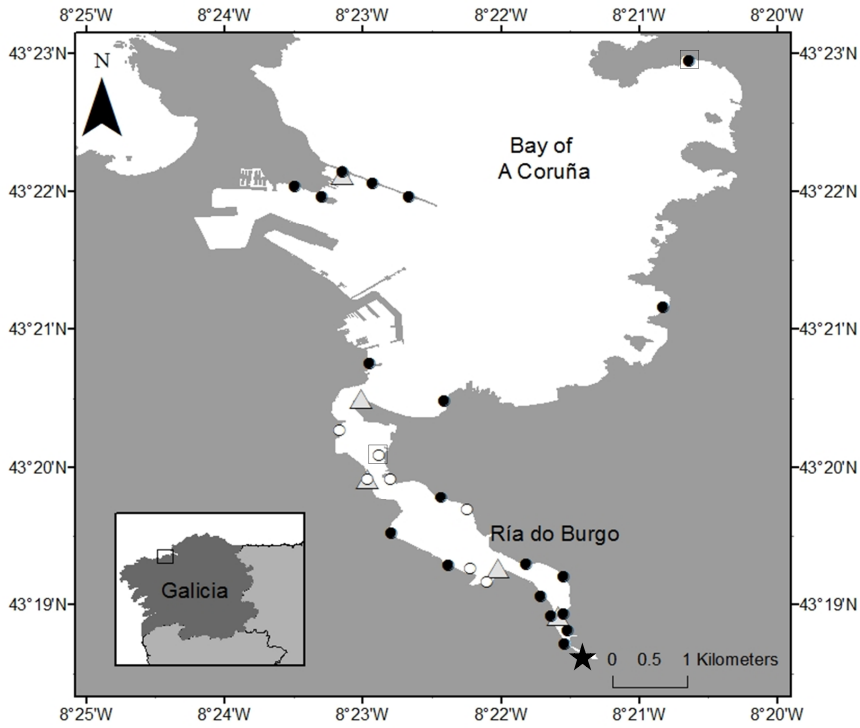

Figure 6.1. Map showing the location of the Ría de A Coruña in Galicia, NW Spain. The enlarged map shows the Ría de A Coruña which is divided in the bay of A Coruña (outer area) and Ría do Burgo (estuarine area). Sampling sites for *Fucus* spp. samples (black circles), *A. nodosum* (open circles) and water (gray triangles) are indicated. The star indicates the reference point to calculate the relative distance of sampling sites to the river discharge point. The open squares mark the Ría do Burgo and Mera sites were *F. vesiculosus*, *A. nodosum* (absent in Mera) and water were sampled every month from October 2010 until January 2012. (Base map: Recursos Marinos y Pesquerías, Universidad de A Coruña).

The seasonal variability of isotopic values in macroalgae and DIN was investigated in samples collected at two sites: one in the estuarine area and one in the outer bay (Fig. 6.1). The estuarine site was located at Ría do Burgo (43°20'N, 8°22'W) in the central part of the estuary and affected by large salinity fluctuations. The oceanic influenced site was located at Mera (43°22'N, 8°20'W), which is a rocky semi-exposed beach near the outer limit of the bay. Sampling included the collection of water samples on a monthly basis from November 2010 to January 2012 (n=13). Macroalgal samples were also collected with monthly frequency from October 2010 to November 2011 (n=13). While *A. nodosum* was found only at Ría do Burgo, *F. vesiculosus* was sampled at both sites.

Interannual changes of macroalgal isotopic values were studied at the same sites that for the seasonal variability, Ría do Burgo and Mera (Fig. 6.1). Samples collected previously along with new samples collected in this study were used. *A. nodosum* and *F. vesiculosus* were used for comparisons at Ría do Burgo. As no seasonal variability in $\delta^{15}N$ was observed at this site, averaged values of different periods were used for both species: Oct-Dec 2010 (n=3), Jan-Nov 2011 (n=10), and July 2013 (n=1). At Mera, only *F. vesiculosus* samples collected in July 2011 and 2013 were used for the comparison. Additional samples of *F. vesiculosus* collected in July 2006 at this site for a previous study were also used.

The spatial variability and the impact of the different nitrogen sources were investigated in water and macroalgal samples collected along the Ría de A Coruña (Fig. 6.1). An arbitrary reference point was established at the river discharge point to compute relative spatial distances between sampling sites along the estuary. Macroalgae were collected at 27 sites at both banks of the ria in July 2013 (Fig. 6.1), while water samples were obtained from 5 sites (Fig. 6.1) between July 2009 to January 2012 (n=14) in order to adequately represent water variability. *A. nodosum* and *F. vesiculosus* were sampled at the same site where available, but when the latter species was not present, any of the two other species of the genus *Fucus* (*F. serratus* and *F. spiralis*) were selected to complete the *F. vesiculosus* gradient as no differences in $\delta^{15}N$ were found among them in previous studies (Viana et al. 2011, Carballeira et al. 2013). Comparisons between values measured in water and macroalgae were made after classification of the sampling points in 0.5-km intervals according to their relative distance from the reference point.

At each sampling site, three macroalgal individuals of each species fixed to the substrate were collected when emerged. Individuals were collected at the meso-littoral dominant zone of each species in the ria (Bárbara et al. 2005). In this area there are two ebb tides every 24 hours, therefore macroalgae of this study might sum up to ~12 hours emerged (submerged) every day depending on their position on the littoral zone and the time of the year.

The apical parts of the specimens (approx. 1 cm) were used for $\delta^{15}N$ determinations. Samples were rinsed with Milli Q water to remove sediments and other material and frozen (-20 °C) before processing. Samples were defrosted and dried (50 °C) until constant weight, before grinding into a homogeneous powder.

Samples of surface water were collected during the ebb tide. Salinity (±0.1, Practical Salinity Scale) and temperature (±0.1 °C) of surface water were measured *in situ* with a portable conductivity meter (YSI Model 30). Water samples for $\delta^{15}N$ determinations were poisoned with $HgCl_2$ (0.05% final concentration) to prevent microbial alteration and stored in tightly caped Pyrex flasks. Subsamples for determination of dissolved inorganic nitrogen concentrations were frozen (-20 °C).

Chemical analysis

Total nitrate (NO_3^-+NO_2^-) and ammonium were determined in the laboratory using segmented flow analysis (Braun-Luebbe AAII) following the procedures of Grasshoff et al. (1983). Sensitivity was 0.05 and 0.04 µM for total nitrate and ammonium, respectively. Precision (se of 3 replicates) was better than 14% of the mean value for both nitrogen species.

The isotopic composition of total nitrate was determined by previous conversion into ammonium and later recovery of ammonium on a solid phase. The procedure is an adaptation of the diffusion method (Sigman et al. 1997) involving the incubation of samples in two steps. In this case, the resulting ammonium was collected on a small disk of glass-fiber filter placed in the gas headspace of the diffusion flask (Slawyk and Raimbault 1995). First, aliquots of the samples were incubated (50 °C, 1 week) in the same collecting flask without cap to reduce the volume and concentrate the total nitrate of the sample. Ashed MgO was added to raise pH above 9.7 to remove ammonia by volatilization. In the second step (50 °C, 2 weeks), ashed Devarda´s alloy was added to the reduced volume sample to convert nitrate and nitrite into ammonium. The high pH (>11) of the mixture ensured also the conversion of ammonium into ammonia gas that was collected on a sterilized glass-fiber disk (Whatman GF/F), acidified with 0.5 ml of 0.25N H_2SO_4 and hooked on a needle fixed to the inner side of the flask cap. Care was taken to ensure that the filter disk did not contact the liquid sample or the flask. This extraction procedure does not allow separation between NO_3^- and NO_2^- therefore the values reported are the combined isotopic signatures of nitrate and nitrite (Ahad et al. 2006). After the second incubation step the disk filters were dried (50 °C) and prepared for isotopic analysis.

The stable isotope composition of ammonium was determined in another aliquot of the water samples by an adaptation of the diffusion method (Holmes et al. 1998).

This method involves gas-phase diffusion as described for the second step of the total nitrate extraction. In all cases, corrections for isotopic fractionation during the whole incubation and diffusion steps were made (Holmes et al. 1998). The measured values of natural abundance of dissolved inorganic nitrogen were retained for further analysis when the ammonium recovery after the diffusion procedure exceeded 45% and isotopic fractionation of internal standards was within 1‰ of values estimated from the empirical equation in Holmes et al. (1998).

Stable isotopes

The natural abundance of nitrogen stable isotopes was determined in macroalgae and water samples (total nitrate and ammonium). For macroalgae, 2.5 mg of dry sample was analyzed to ensure a minimum of 10 µg of nitrogen. For water samples, 1 ml of 4 mM-N $(NH_4)_2SO_4$ was added to each sample during the diffusion phase to ensure the detection limit was achieved. Samples were placed in tin capsules and introduced into an isotope-ratio mass spectrometer (Thermo Finnigan Mat Delta Plus) via an element analyzer (Carlo Erba CHNSO 1108). Isotopic results are expressed in delta notation:

$$\delta^{15}N = [(^{15}N_{sample}:^{14}N_{sample}/^{15}N_{std}:^{14}N_{std})-1] \times 1000$$

where the standard (std) for $\delta^{15}N$ is atmospheric N_2. Precision (se of 5 replicates) was better than 0.05‰ for either IAEA-N-2, IAEA-N-1 or IAEA-NO-3 standards. The coefficient of variation of triplicate sample aliquots was always <2%.

Statistical analysis

The relationships between environmental variables and isotopic values in macroalgae and DIN were analyzed with non-parametric correlation (Spearman ρ). Significance of differences in the water variables (salinity, temperature, total nitrate and ammonium concentrations and their isotopic values) or $\delta^{15}N$ values in macroalgae between different sampling sites were tested with Mann-Whitney (M-W) tests. For the detailed spatial sampling along the salinity gradient, differences were tested between sampling sites grouped in estuarine (salinity <34) or bay sites and between banks, using the above mentioned test.

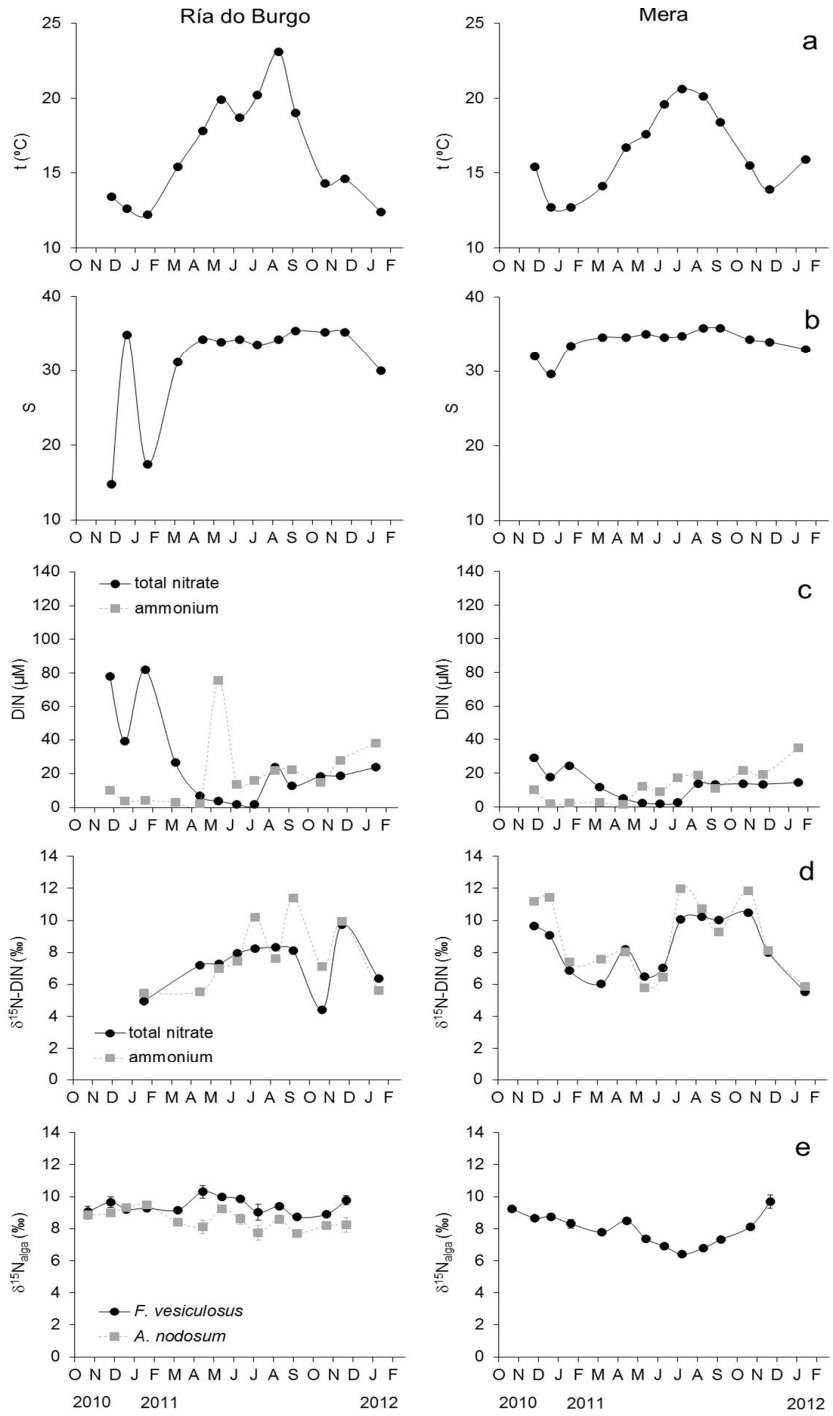

Figure 6.2. Variation in (a) temperature (t, °C), (b) salinity (S), (c) DIN (total nitrate and ammonium, µM), (d) δ^{15}N-DIN (total nitrate and ammonium, ‰) and (e) δ^{15}N in macroalgae (mean±se, *F. vesiculosus* and *A. nodosum*, ‰) at Ría do Burgo and Mera (open squares, Fig. 6.1) from October 2010 until January 2012.

Differences in the isotopic composition of *A. nodosum* and *F. vesiculosus* among different years (interannual variability) were studied by Kruskall-Wallis (K-W) tests and *a posteriori* Dunnett-C tests for multiple comparisons. All the above mentioned tests were performed with SPSS Statistical Software.

Results

Seasonal variability

The main changes observed through the year (Fig. 6.2) were related with decreases in salinity during winter due to the higher volume flow of the river Mero, which affected the site at Ría do Burgo, but had negligible influence at Mera (Fig. 6.2b). Despite the variability observed during these winter months, similar and low concentrations of total nitrate were observed at both sites during the rest of the year. The inverse correlation between total nitrate concentrations and temperature or salinity indicates a minor influence of upwelling compared to river inputs at both sites (Table 6.1). In contrast, ammonium concentrations were uncorrelated with other variables and were lower than those of total nitrate from November 2010 to June 2011, while in summer they were higher and more variable (Fig. 6.2c).

Table 6.1. Spearman ρ correlation coefficients (lower semimatrix) and significance (upper semimatrix) between environmental and isotope composition variables measured at the intertidal stations of Ría do Burgo and Mera (from October 2010 until January 2012). Significant values (P<0.05) appear in boldface. TN: total nitrate, %N: mass percent content in nitrogen of the macroalgal samples, t: temperature, S: salinity.

	t	S	TN	ammonium	$\delta^{15}N_{TN}$	$\delta^{15}N_{ammonium}$	$\%N_{A.\ nodosum}$	$\%N_{F.vesiculosus}$	$\delta^{15}N_{A.\ nodosum}$	$\delta^{15}N_{F.vesiculosus}$
t	-	**0.026**	**0.000**	0.092	**0.038**	0.224	**0.006**	**0.000**	0.112	0.350
S	**0.436**	-	**0.034**	0.434	0.154	0.191	0.879	0.155	0.121	**0.033**
TN	**-0.670**	**-0.417**	-	0.612	0.781	0.920	**0.007**	**0.000**	0.070	0.286
ammonium	0.337	0.160	-0.104	-	0.596	0.744	0.175	0.215	0.572	0.489
$\delta^{15}N_{TN}$	**0.435**	0.307	-0.061	0.117	-	**0.000**	0.576	0.305	0.546	0.268
$\delta^{15}N_{ammonium}$	0.264	0.283	-0.022	0.072	**0.840**	-	0.286	0.511	0.058	0.149
$\%N_{A.\ nodosum}$	**-0.741**	0.049	**0.732**	-0.420	-0.217	-0.400	-	**0.000**	0.306	0.986
$\%N_{F.\ vesiculosus}$	**-0.758**	-0.300	**0.785**	-0.263	-0.235	-0.152	**0.912**	-	0.150	**0.034**
$\delta^{15}N_{A.\ nodosum}$	-0.483	-0.472	0.539	-0.182	-0.233	-0.650	0.308	0.423	-	0.255
$\delta^{15}N_{F.vesiculosus}$	-0.200	**-0.436**	0.227	0.148	-0.253	-0.326	0.005	**0.418**	0.341	-

The isotopic composition of ammonium and total nitrate were positively correlated (Table 6.1). The highest $\delta^{15}N$ values were observed in summer and the lowest during winter, particularly at the oceanic influenced site of Mera (Fig. 6.2d). Despite the similar pattern, total nitrate (but not ammonium) $\delta^{15}N$ was positively correlated with temperature (Table 6.1). Considering all the observations there were not significant differences between sites for any of the water variables considered (M-W test, P>0.05, n=26, and n=23 for isotopic values in DIN).

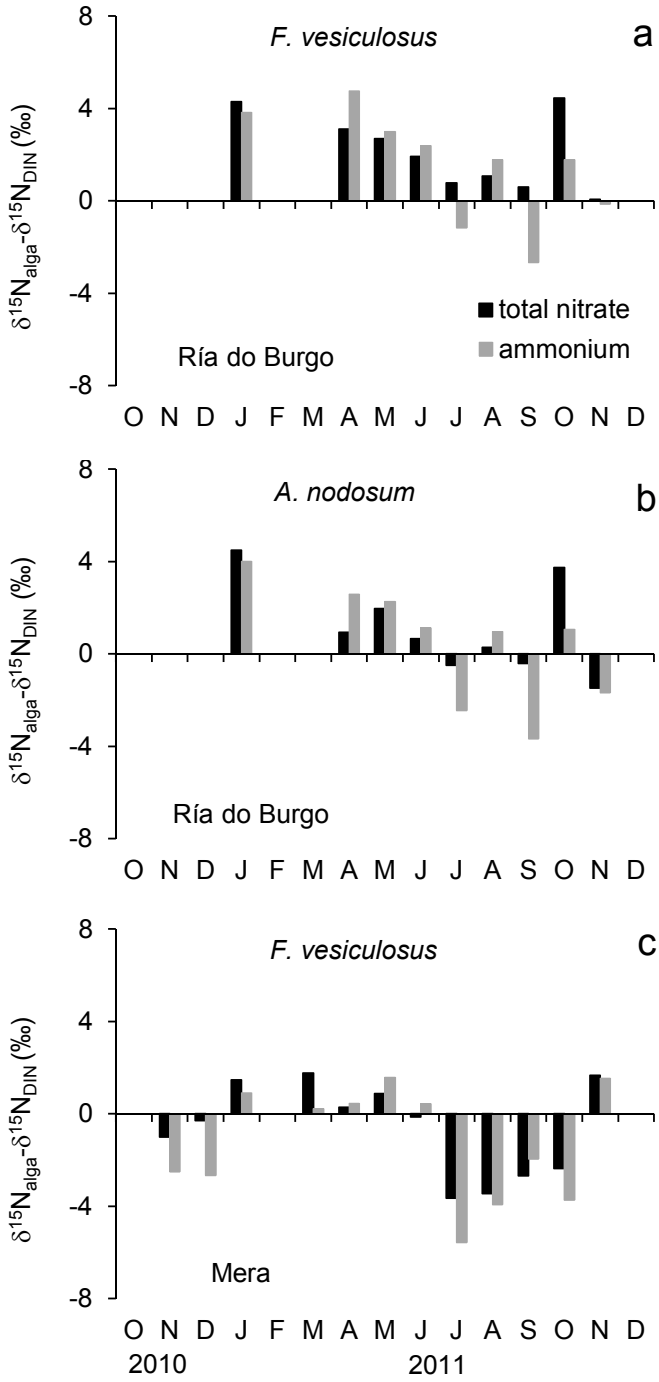

Figure 6.3. Monthly variation (from November 2010 to November 2011) of the difference between the mean $\delta^{15}N$ (‰) of *Fucus* spp. and total nitrate and ammonium at Ría do Burgo (a) and Mera (c) and the difference between the mean $\delta^{15}N$ (‰) of *A. nodosum* and total nitrate and ammonium at Ría do Burgo (b).

The seasonal $\delta^{15}N$ variability observed in macroalgae was lower than in DIN (Fig. 6.2e). Both macroalgal species showed low $\delta^{15}N$ values during spring and summer (minimum in July) and high values in fall and winter (maximum in December 2011). Besides the similar variation along the year, $\delta^{15}N$ of *F. vesiculosus* and *A. nodosum* were not correlated (Table 6.1). On the contrary to what was observed with water samples, $\delta^{15}N$ of *F. vesiculosus* showed higher mean values at Ría do Burgo than at Mera (M-W test, $P<0.001$, $n=26$), although the variability at the latter was higher. This variable was negatively correlated with salinity and positively with the nitrogen content. In contrast, $\delta^{15}N$ values of *A. nodosum* were not correlated with any other variable (Table 6.1).

In Ría do Burgo, mean $\delta^{15}N$ values of *F. vesiculosus* exceeded those of both total nitrate and ammonium for most of the year, except for ammonium in July and September 2011 (Fig. 6.3a). *A. nodosum* followed a similar variation pattern but in this species the $\delta^{15}N$ values were lower than ammonium and total nitrate also in November 2011 (Fig. 6.3b). In contrast, mean $\delta^{15}N$ of *F. vesiculosus* in Mera was only slightly above $\delta^{15}N$ of total nitrate and ammonium between January and June 2011, and also in November 2011, while values in November and December 2010 and between July and October 2011 where lower than those of DIN (Fig. 6.3c).

Interannual variability

Stable nitrogen composition of *F. vesiculosus* and *A. nodosum* showed different interannual variability patterns at both sampling sites (Fig. 6.4). While $\delta^{15}N$ of *F. vesiculosus* did not vary significantly between years at Ría do Burgo, there was a significant increase in 2013 compared to values recorded in 2006 and 2011 at Mera (K-W and Dunnett-C tests, Fig. 6.4a). In turn, *A. nodosum* $\delta^{15}N$ decreased from 2010 to recent years (K-W and Dunnett-C tests, Fig. 6.4b).

Spatial variability

The estuary-ria system of A Coruña showed a marked spatial gradient in salinity but not in temperature (Fig. 6.5a, b). The range of variation of salinity (9 to 34) indicates the differential influence of terrestrial and oceanic waters along the estuarine mixing zone. Annual averaged concentrations of total nitrate (but not ammonium) followed an inverse relationship with salinity, with the highest values near the river end and the lowest values in the outer bay (Fig. 6.5c). Therefore, considering individual paired samples, only total nitrate concentrations were significantly correlated with salinity

(Spearman ρ=-0.684, P<0.001, n=68) indicating the conservative mixing of this nitrogen source between riverine and marine waters along the transect considered.

In contrast, $\delta^{15}N$ of both DIN forms showed a small decrease along the estuary while their corresponding average values were almost constant in the bay (Fig. 6.5d). Overall, $\delta^{15}N$ of ammonium was higher than $\delta^{15}N$ of total nitrate (M-W test, P<0.05, n=68) and both isotopic signatures were significantly correlated (Spearman ρ=0.935, P<0.001, n=68).

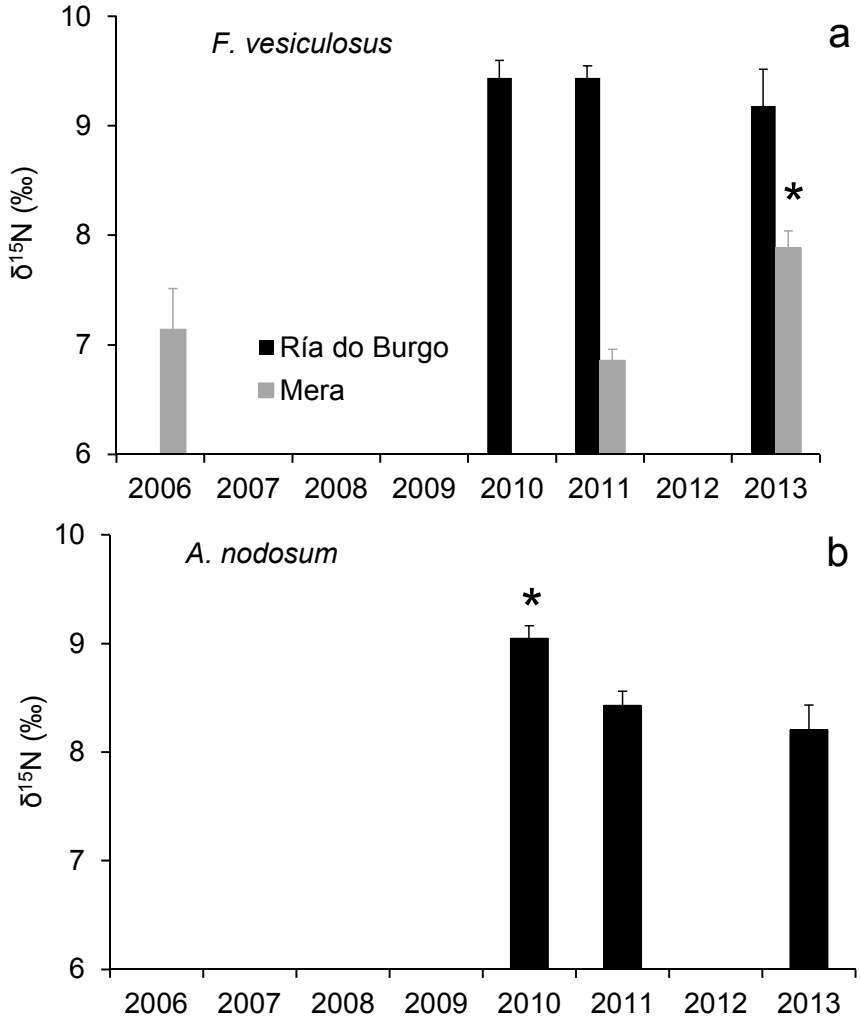

Figure 6.4. Interannual variation of $\delta^{15}N$ (mean±se, ‰) values of *F. vesiculosus* at Ría do Burgo and Mera (a) and *A. nodosum* at Ría do Burgo (b). *: significant differences (Kruskal-Wallis and Dunnett-C tests, *: P<0.05).

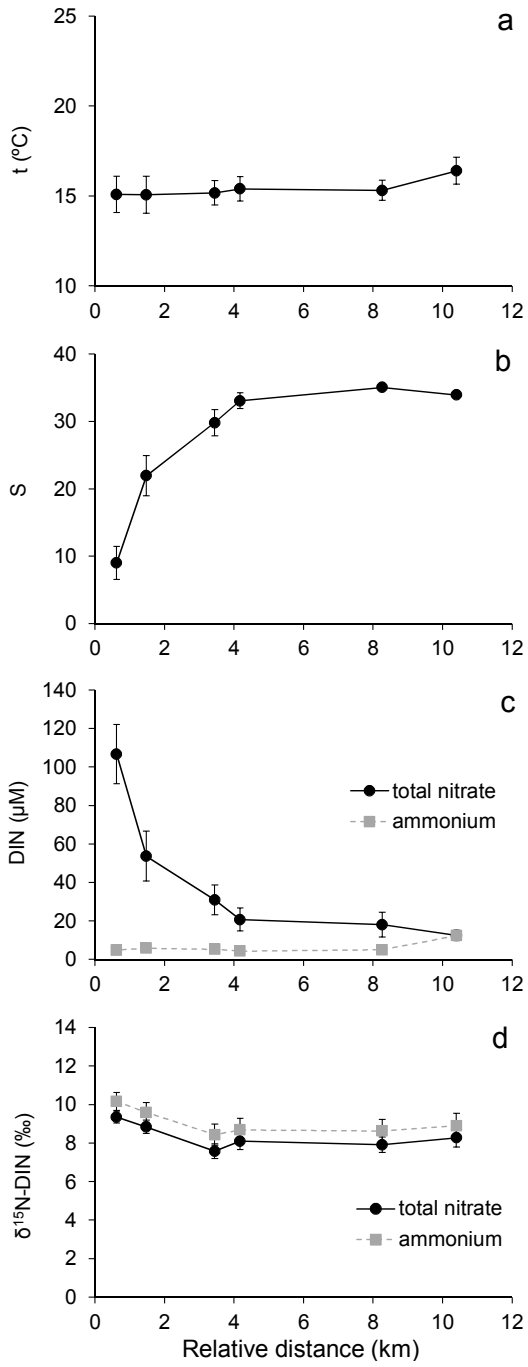

Figure 6.5. Variation of the mean values (±se, from July 2009 to January 2012, n=14) in (a) temperature (t, °C), (b) salinity (S), (c) DIN (total nitrate and ammonium, µM) and (d) $\delta^{15}N$-DIN (total nitrate and ammonium, ‰) along the relative distance (km) from the reference point (Fig. 6.1). Relative distances greater than 5 km (salinity >34) correspond to the bay, while relative distances <5 km correspond to the estuary.

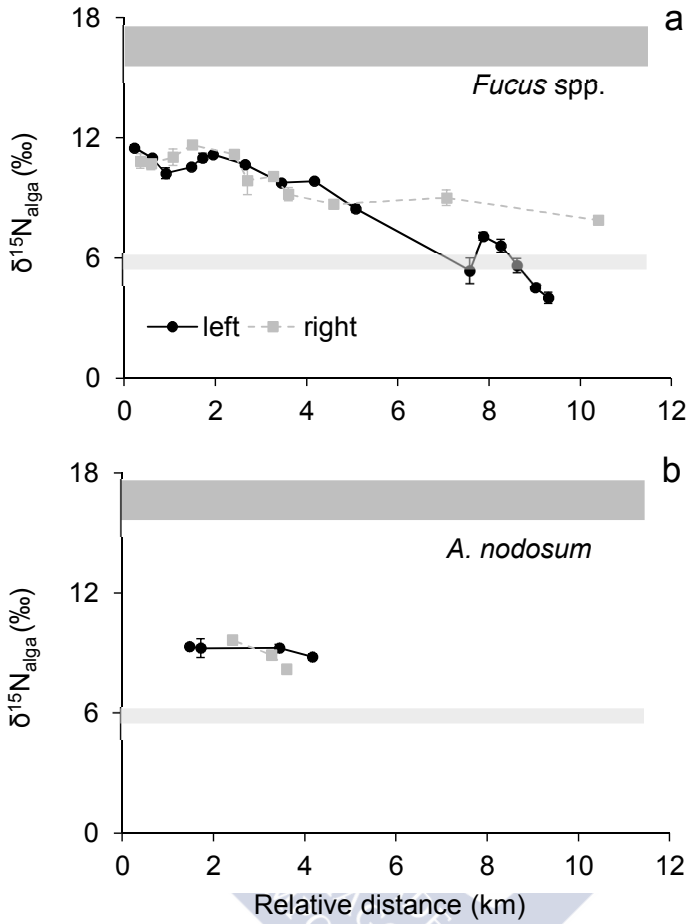

Figure 6.6. Variation of the $\delta^{15}N$ values (mean±se, ‰,) of *Fucus* spp. (a) and *A. nodosum* (b) along the relative distance (km) from the reference point (Fig. 6.1) at the left and right banks in July 2013. Relative distances greater than 5 km correspond to the bay, while relative distances <5 km correspond to the estuary. $\delta^{15}N$ values of end members in the area are represented, the dark gray bar shows the $\delta^{15}N$ values of total nitrate (16.6±1‰, n=3) of a water treatment plant effluent, while the light gray bar shows the $\delta^{15}N$ of total nitrate (6.02±0.28‰, n=24) and ammonium (5.73±0.28‰, n=24) of deep oceanic water (70 m) from the mouth of the Bay of A Coruña.

Macroalgal $\delta^{15}N$ followed a spatial pattern similar to $\delta^{15}N$ in DIN, with values decreasing from the inner estuary to the outer bay (Fig. 6.6). Macroalgae from the estuary had nitrogen isotopic values closer to the $\delta^{15}N$ of an effluent of a water treatment plant. While isotopic values within the bay decreased towards the isotopic values of deep oceanic water from the mouth of the Bay of A Coruña (Fig. 6.6). $\delta^{15}N$ values of *Fucus* spp. from the estuary were significantly higher than those from the bay

(M-W test, P<0.001, n=88). In the latter zone, there was also a significant difference between the $\delta^{15}N$ values of *Fucus* spp. collected at the different banks, with specimens from the right bank more enriched than those from the left bank (M-W test, P<0.001, n=33). However, there were no significant differences between banks in the isotopic composition of specimens of *Fucus* spp. or *A. nodosum* inside the estuary (M-W tests, P>0.05, n=55 and n=29 for *Fucus* spp. and *A. nodosum*, respectively).

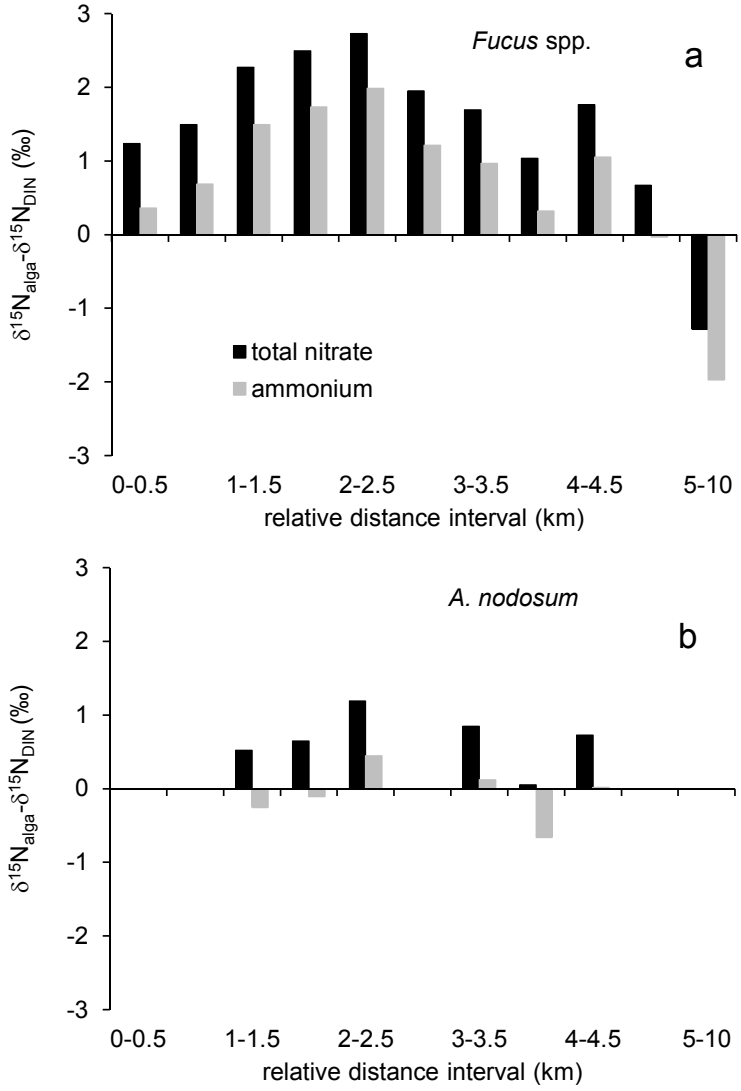

Figure 6.7. Variation of the difference between the mean $\delta^{15}N$ (‰) in total nitrate and ammonium and *Fucus* spp. (a) and *A. nodosum* (b) at increasing relative distance intervals (0.5-km bins) from the reference point (Fig. 6.1). The relative distance interval greater than 5 km correspond to the bay, while distance intervals <5 km correspond to the estuary.

In general, macroalgae were enriched in ^{15}N relative to DIN along the study area, with the exception of the depleted values measured for *Fucus* spp. in the outer bay (Fig. 6.7a). The enrichment increased from the river end (0 km in the reference distance scale) to the center of the estuary (2.5 km) and decreased thereafter towards the outer bay (up to 10 km). This enrichment was higher for *Fucus* spp. than for *A. nodosum*, the latter showing even ^{15}N depletion relative to ammonium at some sites (Fig. 6.7b). When compared in these 0.5-km intervals, $\delta^{15}N$ of *Fucus* spp. was significantly correlated with isotopic values of ammonium or total nitrate (Spearman $\rho=0.864$, $P<0.001$, $n=11$, for both total nitrate and ammonium). In contrast, no significant correlation was found between isotopic values of DIN and *A. nodosum* because this macroalgae is restricted to the estuarine site with less isotopic variability in algae and DIN.

Discussion

Correspondence between $\delta^{15}N$ in algae and water

The isotopic values of native fucoid species were not correlated with concurrent isotopic values of DIN (Table 6.1). This result has been also reported in other studies and was attributed to isotopic fractionation processes (Deutsch and Voss 2006, Raimonet et al. 2013) or to the rapid mixing of different nitrogen sources in estuaries (Derse et al. 2007). Fractionation can be observed in almost all physical processes and chemical reactions involved in the cycle of nitrogen (Mariotti et al. 1981). On the other hand, water mixing is an effective mechanism to dilute isotopically enriched DIN discharged to coastal waters (Ahad et al. 2006, Raimonet et al. 2013). Periodic mixing events (as in most tidal regimes) would enhance high frequency variability in the isotopic composition of DIN that may not be tracked by the macroalgae.

The net isotopic fractionation of macroalgal N can be defined as the result of all processes involved in the incorporation of nitrogen to macroalgal tissues, i.e. uptake from the water, assimilation into organic compounds, storage and release of dissolved forms. Fractionation during uptake leads to depletion of $\delta^{15}N$ in the algal tissues compared to DIN (Pennock et al. 1996). However, there is some controversy about the importance of this type of fractionation for macroalgae, as there are examples of no fractionation (Cohen and Fong 2005, García-Sanz 2009) or significant fractionation for some compounds (Kaldy 2011). In our study, fractionation during uptake may have been limited to the few cases with macroalgal $\delta^{15}N$ lower than $\delta^{15}N$

in DIN sources, as observed for *Fucus* spp. in the bay, both during late summer and fall (Fig. 6.3) and for the spatial distribution study in July 2013 (Fig. 6.7). Apart from these cases, the lack of significant correlations between the $\delta^{15}N$ of macroalgae and DIN concentrations suggests that uptake fractionation was not the main mechanism explaining $\delta^{15}N$ in the studied macroalgae.

Fractionation can also result from the release of nitrogen from the macroalgae. In this case the macroalgae would be more enriched than DIN in the surrounding water, as they would preferentially release the light isotope (Wada et al. 1975). Release of both inorganic and organic compounds has been detected in several macroalgal species (Fong et al. 2004, Tyler and McGlathery 2006). It was attributed to a response to different stressors (Young et al. 2009) and particularly to emersion (Kim et al. 2013). As far as we know, there are no reports of nitrogen release in the species considered in this study, but our results suggest that this process is enhanced in the zone with large changes in salinity. The frequent changes in salinity experienced by the central part of the estuary (error bars, Fig. 6.5) would act as an stressor agent.

Nitrogen content of macroalgae is mainly related with total nitrate concentrations (Table 6.1), as this nutrient is the preferred DIN source for growth (Pedersen and Borum 1997). Therefore the changing relation between macroalgal and DIN isotopic values is not expected to be related with the preference for other dissolved nitrogen forms, as DON or NH_4^+. Even though ammonium was preferred, the similar isotopic values of both DIN components would not affect the $\delta^{15}N$ values in macroalgae. Besides, there was not DIN depletion in the area, so the preferred use of other dissolved forms might not be expected (Tyler et al. 2005).

The variability in the isotopic enrichment of macroalgae relative to DIN found in this study implies that, at any given time, both compartments are in different states of isotopic exchange. These species have slow apical growth rates, varying from 2.2 to 1.4 cm mo^{-1} in *F. vesiculosus* (Viana et al. in review b) to 1.6 cm mo^{-1} in *A. nodosum* (Viana et al. in press). Therefore, the 1-cm sample used in this study corresponds to a 14-day and 21-day exposure time of *F. vesiculosus* and *A. nodosum* respectively (Viana et al. in press, in review b). Besides, this would imply the correspondence of isotopic values in macroalgae with isotopic values of DIN of previous months, but this was never observed in this study (Fig. 6.2d, e). Therefore, correspondence between $\delta^{15}N$ values of these macroalgae and DIN cannot be expected because the

$\delta^{15}N$ in these macroalgae is the result of all nitrogen sources taken up, assimilated and released during a relatively long time period, and the $\delta^{15}N$ of DIN in estuaries shows large variations due to mixing of the different water types.

Temporal and interannual variability in algal $\delta^{15}N$

Intrinsic factors may have partly influenced on the variability of macroalgal $\delta^{15}N$ along the year. *F. vesiculosus* population from Mera display high growth rates during summer (Viana et al. in review b), when a decrease in $\delta^{15}N$ in their apical tips was recorded (Fig. 6.2). *Fucus* species have also been shown to have marked differences in the activity of nitrate reductase and nitrogen storage patterns at different times of the year (Young et al. 2007). Therefore, they show the lowest internal nitrogen concentrations in summer due to the dilution effect caused by the mobilization of nutrients to sustain growth (Villares et al. 2013). In our study, the negative correlation between the nitrogen content of both macroalgae and temperature accounts for the decrease of internal nitrogen in summer (Table 6.1). The mobilization of nitrogen for growth may derive in some fractionation, thus producing low $\delta^{15}N$ values in the apical growing tips during summer due to the preferential use of ^{14}N, and leading to changing relations with $\delta^{15}N$-DIN in the different months (Figs. 6.2e and 6.3). Nevertheless, the recorded seasonal variability in $\delta^{15}N$ of the studied macroalgae is very low when compared to other taxonomic groups (Pruell et al. 2006).

The perennial character and the long turnover times of these species make them suitable biomonitors for interannual comparisons in the study region, as data on nutrient concentrations are scarce for estuarine sites (e.g. Varela and Prego 2003, Bode et al. 2014). In this way, the decrease in $\delta^{15}N$ of *A. nodosum* between 2010 and 2013 (Fig. 6.4) is consistent with the improvement in the wastewater treatment and disposal facilities near large urban areas and with the general pattern observed at the coast of Galicia during the last decade (Viana et al. 2011). Contrary to this general pattern, the increase in $\delta^{15}N$ of *F. vesiculosus* observed at Mera suggests a recent increase in local nitrogen discharges at this beach, supporting a small permanent rural population (Viana and Bode 2013) but affected by the visits of a large number of tourists during summer. Nevertheless, the use of these species on a long-term monitoring basis needs further understanding of local variability. If environmental variables can derive in different isotopic values in macroalgae on a monthly scale, differences might also be expected on an annual basis. For instance an enrichment of $\delta^{15}N$ in some macroalgae was related with warmer temperatures or higher intensity

of upwelling regimes (Baeta et al. 2009, Viana and Bode 2013). Therefore isotopic changes cannot be strictly related with changes in N sources, but also with changing environments.

Spatial variability in algal $\delta^{15}N$

Notwithstanding the temporal variability of isotopic values in macroalgae (Fig. 6.2), the variability between locations was in general much higher (Fig. 6.6). The highest $\delta^{15}N$ values were recorded near the river end of the estuary and the lowest in the bay, following the trend of average $\delta^{15}N$ values of DIN. The isotopic composition of *Fucus* spp. allowed identifying two clearly distinct environments: the bay and the estuary. This result is consistent with previous studies that stress the high marine influence in the bay of A Coruña (Cabanas et al. 1987, Varela et al. 1994, Bode et al. 2014), while the low flow of freshwater from river Mero restricts the estuarine influence to the small Ría do Burgo (Bode et al. 2014). The higher influence of the river inputs to the estuary, as indicated by low salinity and high total nitrate concentrations (Fig. 6.5), may have contributed to the enriched macroalgal $\delta^{15}N$ values within the estuary as $\delta^{15}N$ in DIN was also enriched in low salinity waters. Previous studies also showed that *Fucus* spp. specimens collected from the inner parts of the Galician rias had higher $\delta^{15}N$ values than specimens collected from marine waters (Bode et al. 2006, Viana et al. 2011).

Within the bay, there was a decreasing gradient of isotopic macroalgal values from the estuary mouth to the ocean, but interestingly, significant differences between banks were detected (Fig. 6.6). Macroalgal $\delta^{15}N$ values in the right bank, surrounded mostly by rural populations, were ~8‰, while those in the left bank, occupied by the large urban development of the city of A Coruña, were from ~4 to 6.6‰. Although diffuse nutrient discharges cannot be discarded, due to the scattered housing development in the area, previous studies showed that urban influence in this ria leads to high nutrient concentrations in the proximities of the harbor at the left bank of the bay (Varela and Prego 2003), so the influence of high DIN concentrations on isotopic values might be expected for macroalgae in the left bank. Apart from the possible existence of point effluent discharges on the right bank, the isotopic values of macroalgae on the left bank are even lower than the $\delta^{15}N$ values of DIN from the deep oceanic waters in the area (Fig. 6.6a). For this reason and because DIN sources did not show marked changes in $\delta^{15}N$ in the bay, we conclude that enhanced fractionation due to preferential uptake of ^{14}N may have caused the low $\delta^{15}N$ in macroalgae in the harbor influenced area.

Implications for monitoring anthropogenic N

While it is generally agreed that $\delta^{15}N$ of brown macroalgae can discriminate between anthropogenic and natural sources of nitrogen (Gartner et al. 2002, Savage and Elmgren 2004, Deutsch and Voss 2006, García-Sanz 2009, Carballeira et al. 2013), this study provides new insights on net fractionation processes occurring in fucoid species. The results of the present study highlight the existence of sources of variability in macroalgal $\delta^{15}N$ other than the DIN sources. Although high $\delta^{15}N$ values were measured in macroalgae from areas affected by continental inputs, and likely large influence of anthropogenic nitrogen, variability due to isotopic fractionation appears to be more important than initially considered. These fractionation processes may have not been previously detected due to the scarcity of studies relating $\delta^{15}N$-DIN and native macroalgal values (Raimonet et al. 2013, Viana and Bode 2013).

Our results support the use of brown algae as biomonitors of nitrogen sources over long spatial (km) scales, as their $\delta^{15}N$ integrate the small scale variability in nitrogen sources. Although for an use of these macroalgae as quantitative biomonitors further understanding of the fractionation processes is needed, they can be reliably used to distinguish impacted and reference sites at a local or regional scale.

They can be used to do studies in a long-term basis. However, the results show they are not reliable biomonitors of short term changes in ambient DIN, as their $\delta^{15}N$ was not correlated with DIN isotopic variability year round. This integration avoids the large variability observed in $\delta^{15}N$ of green and red algae that rapidly respond to changes in the sources of nitrogen, as observed with residual waters (Gartner et al. 2002). As they are perennial and they show apical growth, the growing period of a segment can be easily determined (Viana et al. in press, in review b). Nevertheless, when the study is focused on detecting interannual variability, sampling might be done at the same season every year or average annual values of the different seasons must be computed, as variable fractionation processes can lead to a misinterpretation of $\delta^{15}N$ values.

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General Discussion

The research carried out during this thesis has brought new insights for the use of *Fucus* spp. and *A. nodosum* as biomonitors of the impact of anthropogenic N sources on littoral ecosystems. The results of this research can be applied to specific studies aiming to quantify anthropogenic impacts using either native populations or experimental incubations of these species, the latter applicable when these species are not naturally present in the impacted area. The envisaged application goes beyond previous studies demonstrating the link between anthropogenic pressures and isotopic values in macroalgae (McClelland and Valiela 1998a, García-Sanz et al. 2010, Carballeira et al. 2013). Specifically, as N is also an essential element for macroalgae, the present research addresses the understanding of the variability of N sources and impacts on macroalgae at different spatial and temporal scales, thus providing a template for a better exploitation of stable isotopic values in monitoring studies.

Determination of biogeographic gradients and relation with urban aggregations

Biomonitoring N with fucoid macroalgal $\delta^{15}N$ has the advantage of providing comparable data on a regional scale basis due to their wide range of distribution (Viana et al. 2011). However, in coastal environments the nitrogen inputs come from several sources that can vary at regional spatial scales. When comparing different environments over a large region, as in the case of NW coast of Spain, a geographic latitudinal effect of the natural variability on the isotopic values of macroalgae needs to be considered. Local influence of rivers, salt marshes and other environments affecting N dynamics on the continental side, and marine effects caused by features as coastal upwelling produce variable mixtures of natural N sources that can affect the interpretation of anthropogenic sources at regional scales. Thus, obtaining comparable estimations of anthropogenic impacts along wide coastal ranges requires not only on the presence of the selected biomonitors in different locations, but also the understanding of the geographic factors affecting natural isotopic values at large coastal ranges. For instance, the different reference values given in studies with the selected species (Table 1.1) or with other macroalgal species (Dailer et al. 2010) is highly variable, and values from 2.1 to 5.9‰ were observed in the different areas. Therefore, it can be deduced that the geographical variability of natural N processes of both natural inputs and reactions within the N cycle itself may affect the baseline levels of macroalgae.

In the study area, macroalgae are enriched in heavy isotopes at sites of large influence of upwelling while they are depleted where the upwelling has lower influence. Latitudinal variability was also observed in other macrophytes (Christiaen et al. 2013), and it was related with upwelling processes in some biota in South Africa (Hill and McQuaid 2008). This variability can be predicted to some extent thus facilitating the comparison of isotopic signatures of organisms collected in regions of changing N inputs.

Besides the natural gradient, the anthropogenic influence also accounted for part of the variance observed in $\delta^{15}N$ for both species, with an increase of $\delta^{15}N$ with the size of the urban population. Previous studies in Galicia have shown that enriched macroalgal $\delta^{15}N$ values could be related to local anthropogenic N inputs (Bode et al. 2011b) particularly inside the rias (Viana et al. 2011), but no quantitative estimations of the variability in N sources were made in this or other areas with these species. Nevertheless, isotopic signatures in macroalgae are not simply related to the size of the human population, as for instance, the variability in small populations is very high while mean values observed near large cities (>15,000 inhabitants) are relatively less variable. The reasons for isotopic variability observed in macroalgal samples close to small urban nuclei is difficult to explain due to the absence of a direct relationship between $\delta^{15}N$ of concurrent macroalgal and water samples. Both external and internal factors could explain these differences, as the different wastewater treatments applied, their efficiency, or the influence of macroalgal metabolism.

Therefore, from this content block some recommendations can be extracted when establishing monitoring protocols or when comparing isotopic values at large regional or latitudinal scales, from the same or from different studies. First, a good knowledge of local or regional natural factors affecting isotopic signatures is needed for the interpretation of the results, as the existence of upwelling processes, or high N_2 fixation in the area (as in Lamb et al. 2012). Second, it is also necessary to consider that enriched isotopic values are not simply related with higher anthropogenic pressure.

Applying the use of N isotopic values in macroalgae to retrospective studies: a solution for long-term monitoring programs?

Long-term monitoring is needed to track the ecological status of ecosystems in time (Koslow and Couture 2013) or to contextualize current observations. However, obtaining reliable and long-time series generally requires a careful sampling plan implemented during decades at sites sensitive to anthropogenic influence. Consequently there are only a few examples of time series using stable isotopes and only for selected sites (e.g. Viana et al. 2011). Therefore, there were great expectations on using the old-growth parts of these macroalgae for retrospective studies, which would help to interpret the impact of anthropogenic derived N (Savage and Elmgren 2004, Raimonet et al. 2013, Carballeira et al. 2014) or other contaminants (Stengel et al. 2005, Heldal and Sjøtun 2010). Moreover, this approach would allow reducing the sampling effort in monitoring programs as it would allow reducing sampling frequency (Carballeira et al. 2014). However, when using these species as retrospective biomonitors two main assumptions remained untested until now: i) to feasibly relate macroalgal segments with different exposure periods (months, years), and ii) to verify if the macroalgal physiology could alter the isotopic signature of non-growing segments.

To relate one macroalgal segment with a particular exposure period, two approaches can be done with the selected species. First, the number of bifurcations in *F. vesiculosus* or the number of gas bladders in *A. nodosum* could be used for dating different growing periods (Savage and Elmgren 2004, Stengel et al. 2005). These markers of age produced different results. In contrast to previous studies (Savage and Elmgren 2004) we could not obtain a clear relationship between the number of bifurcations in *F. vesiculosus* and the length or age of the individuals (Fig. 7.1). The number of bifurcations for this species varies mainly with exposure and salinity rather than with age (Jordan and Vadas 1972, Kalvas and Kautsky 1993, López-Rodríguez et al. 1999). Therefore the level of branching of this species cannot be used as a proxy for age and therefore for retrospective studies of past nitrogen sources using stable isotopes. On the contrary, our study confirmed the annual appearance of gas bladders in *A. nodosum* after the first year of life (Niell 1979). Because of this feature, the gas bladder of this species could be used for dating yearly segments of their thallus.

On the other side, the growth curves of the species can be estimated. This would be particularly important for *F. vesiculosus* due to the absence of a clear relationship between the number of dichotomies and age. The growth curves for different *F. vesiculosus* and *A. nodosum* populations obtained in Chapters 3 and 4 were intended for application to retrospective studies. With these curves we are able to estimate the time required for individuals from these populations for reaching a specific length, and these estimations can be extended to populations living in similar environments (i.e. estuarine or semi-exposed sites). Because these species have apical growth, if the isotopic composition of the growing segment remains unchanged after growth we could estimate the N sources at the time of growth by analysing different segments of the thallus.

For the second assumption, the enrichment experiments described in Chapter 5 demonstrated that all sections of the thallus of both species take up N from the ambient water when submerged. Even when there was no transport of N along the thallus, the results show that this uptake would affect the $\delta^{15}N$ of non-growing sections. The differential values along the thallus observed in previous studies cannot be a direct consequence of the exposure to different $\delta^{15}N$-DIN values during previous years. Therefore, it is not possible to obtain feasible estimates of past N sources from the $\delta^{15}N$ of different sections of the fronds of *A. nodosum* or *F. vesiculosus*.

Even though long-term monitoring programs cannot be based on the retrospective analysis of individuals, the growing tips can still be used for the establishment of these programs (Viana et al. 2011). Due to the different values observed along the thallus, it is important to limit the use to the apical tip and also specify the length of the segment, as this may influence the exposure period and hence the interpretation of the results. From the growth curves estimated in Chapters 3 and 4, the reflected period by the apical tips with different lengths can be calculated (Table 7.1). Until now, most studies using these species for monitoring purposes have considered the use of the growing tips, although different length segments have been used (Table 1.1).

Moreover, the estimated growth rates are also age-dependent in these species (Chapters 3 and 4). Therefore the total length of the individuals used should be similar, in order to guarantee that the segments considered are reflecting the same growing periods. Individuals of 10 and 60 cm long for *F. vesiculosus* and *A. nodosum* respectively

would be adequate as at that time they show exponential growth rates and they have not reached the length when the probability of suffering breakages is higher.

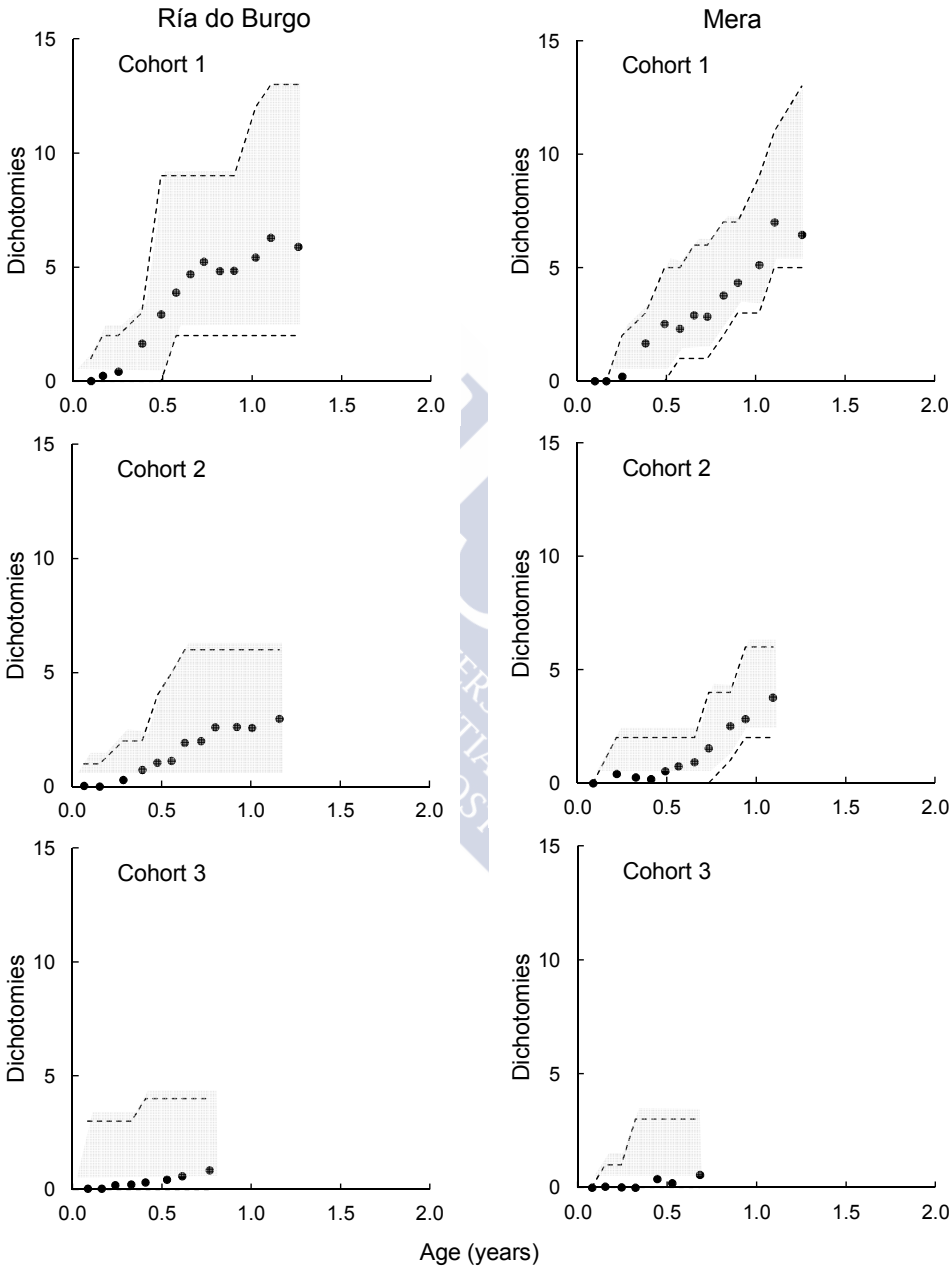

Figure 7.1. Mean (dots) and range (shaded area) of the number of dichotomies in the thallus observed for the three cohorts of *F. vesiculosus* at Ría do Burgo and Mera (Chapter 3).

Table 7.1. Growth time (days) reflected by individuals of 10-cm long of *F. vesiculosus* and 60-cm long of *A. nodosum* from Ría do Burgo depending on the length of the apical segment considered. The estimated time is based on the growing curves obtained for cohort 1 in *F. vesiculosus* (Chapter 3) and for *A. nodosum* at this site in 2010 (Chapter 4).

	F. vesiculosus	*A. nodosum*
1 cm	14	21
3 cm	42	63
5 cm	71	106
10 cm	177	222

Applying the use of $\delta^{15}N$ in macroalgae for the study of the local influence of N sources. The case study of the Ría de A Coruña

Differential $\delta^{15}N$ values in macroalgae in monitoring studies at local scales have been attributed to the differential impact or mixing of the sources. The laboratory experiments and the case study at the Ría de A Coruña highlighted the importance of external and macroalgal intrinsic factors for the interpretation of macroalgal $\delta^{15}N$ values at local scales.

Among external factors some clues about where, when and how often to do the samplings can be deduced. The results revealed that spatial variation of $\delta^{15}N$ in macroalgae was of paramount importance, even when changes in isotopic values of DIN were not significant at this scale. Both macroalgae and DIN reflected that the estuary (Ría do Burgo) was more impacted by isotopically enriched N than the outer bay of A Coruña. This variability suggested the larger influence of anthropogenic N in the estuary while marine N would dominate in the bay. Therefore, to account for the general status of the ria or estuary a minimum of two sampling sites are needed: one in the inner part, where salinity is <34, and other site where salinity is >35. For selecting the position of these sites, the circulation of the water must also be considered.

In contrast, isotopic DIN values varied mainly with time, at least at the seasonal scale examined, while macroalgal $\delta^{15}N$ variation was not that high. These differences might be explained as a direct consequence of the integration times necessary to

acquire the $\delta^{15}N$ signature of DIN for both macroalgal species (Chapter 5). When establishing monitoring protocols, this variability might also be considered. Due to the low seasonal variability observed, samplings can be restricted to a few months, or at least one month along the year. Taking into account the growth rates of the species, a 6-month periodicity will be ideal for covering the annual variability at a particular site. But if sampling is done at an annual basis (as in Chapter 6), the same sampling month should be considered. Taking into account the observed variability, sampling surveys should be established between spring and summer, when higher isotopic values are observed in estuarine sites and most oceanic influenced sites respectively.

Besides the use of natural populations, these macroalgae can be used in transplantation or laboratory experimental incubations with different water origins to determine potential impacts of different N sources (Chapter 5). Apart from the previous recommendations that might be also considered in these studies, in this case, it is important to consider the nitrogen turnover rates and equilibration times of the tips for these species when establishing the duration of the incubations. The $\delta^{15}N$ of the tips of macroalgae integrate N sources in the ambient water over scales of 15 days (*F. vesiculosus*) and up to 6 months (*A. nodosum*). With lower incubation times the results will not reflect the real impact of the ambient N sources.

Concerning the intrinsic factors of macroalgae, the absence of a direct and conservative relationship between macroalgal and water $\delta^{15}N$ complicates the quantitative interpretation of isolated macroalgal $\delta^{15}N$ (as shown in Chapter 2). In Chapter 6 this relationship was shown to be variable in time and space, although a concordance was observed when values were averaged over a large spatial area. The integration times necessary for macroalgae to acquire the $\delta^{15}N$ signature of DIN might explain this difference. This is due to the fact that they are not reflecting the same N composition at a particular time. Our results illustrate the need to address the variability of isotopic signatures along a gradient. Many studies using $\delta^{15}N$ values in macroalgae to detect the area of influence of point source effluents used gradients that reported decreasing values with distance from the emission source (Costanzo et al. 2001, Savage and Elmgren 2004, García-Sanz et al. 2010, Carballeira et al. 2013). The problem arises when diffuse or unknown sources are present in a large salinity gradient, as shown in Chapter 6. In these cases, repeated studies of $\delta^{15}N$ in the macroalgae along the main spatial gradient are required to account for the unknown variability in the N sources.

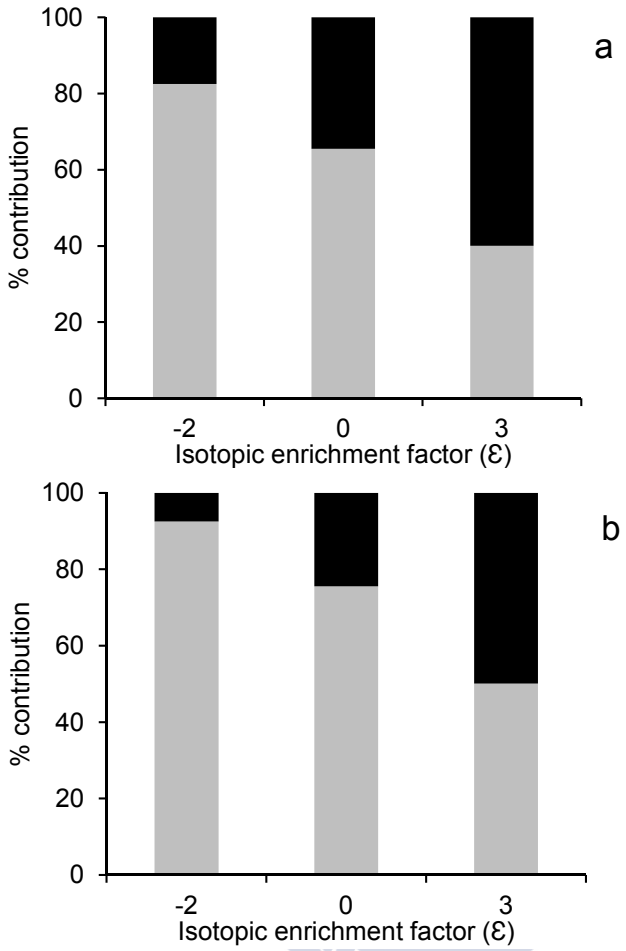

Figure 7.2. Percent contribution of anthropogenic (black bars) and marine nitrate (gray bars) to *Fucus* spp. (a) and *A. nodosum* (b) $\delta^{15}N$ values at a site at Ría do Burgo. The contribution was estimated from a mixing model with two end-members: $\delta^{15}N_{ma} = f_a\delta^{15}N_a + (1-f_a)\ \delta^{15}N_m - Ɛ$, where the $\delta^{15}N_{ma}$ is the isotopic composition of macroalgae, f_a and $1-f_a$ the contributions of anthropogenic and marine sources respectively, and $\delta^{15}N_a$ and $\delta^{15}N_m$ the anthropogenic and marine isotopic values of total nitrate. The $\delta^{15}N_a$ was obtained from the mean value of several urban wastewater samples of the region (17.8±0.6‰, n=7). And the $\delta^{15}N_m$ is the mean value of deep oceanic water from the Bay of A Coruña (6.02±0.28‰, n=24). Each bar represents the % contribution of both sources estimated using 3 hypothetical isotopic enrichment factors (Ɛ).

Besides integration times, isotopic fractionation during N metabolism may be more important to determine the observed $\delta^{15}N$ composition than temporal or even spatial variability. When estimating the fractional contribution of anthropogenic and natural sources it is generally assumed that these macroalgae do not exhibit isotopic fractionation (e.g. Savage and Elmgren 2004). If the fractionation factor is

not known, the isotopic values in macroalgae can lead to misinterpretation of the contribution of anthropogenic sources (Fig. 7.2). Because of the higher enrichment values in macroalgae compared to water observed in Chapter 6, the possible influence of fractionation during N uptake can be discarded for these species, as it was shown for other species (Cohen and Fong 2005, García-Sanz 2009). The mechanism causing this positive fractionation in the macroalgae may be related to nutrient release (Umezawa et al. 2007). This process is well studied in animals as is the base of food webs studies (Montoya 2008) but it has been poorly studied in macroalgal species. Although the existence of organic and inorganic nutrient release has been observed in some macroalgae (Tyler et al. 2001, Naldi and Wheeler 2002), little is known about the isotopic fractionation processes or the environmental factors involved.

The isotopic values of *A. nodosum* and *Fucus* spp. growing at the same site, although correlated, were different. This result suggests the existence of differences in the fate of N taken up for the different species. These differences may be related to the uptake process or, more likely, to the subsequent release of part of the nitrogen after incorporation into the algal tissues. Such differential processing of N among species would have important implications for the use of a particular species as biomonitor of N loadings, especially when comparing results between systems or regions of the same system where species composition differs. However, species differences in nitrogen uptake may be useful for understanding macroalgae responses to anthropogenic sources and elevated N concentrations. From these results we can conclude that the integration times of these species are particularly appropriate to differentiate chronic pollution from point discharges that may have little impact on brown macroalgae.

Considering the current limitations highlighted in the studies included in this thesis, future prospects can be disclosed in two main aspects. First, further studies aiming to understand the link between species-specific N metabolism and its link with isotope fractionation are needed to fully interpret the current data. Second, the existence of fractionation during release of internal N needs to be ascertained as it would explain the enrichment of macroalgal tissues relative to the water DIN.

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General Conclusions

1. The $\delta^{15}N$ values of A. nodosum and F. vesiculosus are significantly influenced by the isotopic signature of both anthropogenic (wastewater) and natural sources (upwelling). The interpretation of results at large geographic scales including a gradient of upwelling requires previous knowledge of the influence of upwelling on $\delta^{15}N$ at each site.

2. The $\delta^{15}N$ of *A. nodosum* and *F. vesiculosus* increase with the size of human population nuclei for populations <15,000 inhabitants but with large associated variability, whereas for larger population sizes the isotopic values are unrelated with the size of the population. This suggests the existence of differences in the efficiency of nitrogen removal from wastewater of small cities and villages or differences among water treatments.

3. The starting populations of *F. vesiculosus* after experimental denudations were established through the recruitment of three different cohorts during a 17-month period. The growth curves obtained followed a logistic pattern and were different between cohorts established at different times during the year, and between sites (semi-exposed and estuarine). Overall, a 1-cm tip would represent the growth of the previous 14 days for an individual at an estuarine site.

4. The recruitment of *A. nodosum* in experimentally denudated areas was a slow process and only one cohort was detected during the 26-month study period. When studying adult populations, differences among growth curves estimated for different localities were observed, highlighting the importance of local factors for the growth of this species. Overall, at an estuarine site, a 1-cm tip would approximately represent the individual´s growth of the previous 21 days.

5. The basal and old growth parts of the fronds of *A. nodosum* and *F. vesiculosus* cannot be recommended for using in retrospective isotopic studies because they maintain N uptake capabilities during the individual´s life. The solely use of growing tips is recommended instead as they show apical growth and no transport was observed along the thallus.

6. Isotopic changes in macroalgae cannot be directly related with $\delta^{15}N$ of DIN sampled concurrently at regional or local scales. This is because *A. nodosum* is able to integrate the DIN variability up to a 6-month period if the macroalga is all time submerged, while *F. vesiculosus* integrates DIN variability of a 15-day period. This is of special importance in laboratory or transplantation studies.

7. The use of macroalgal $\delta^{15}N$ for doing interannual comparisons is appropriate as they are perennial and they integrate nitrogen variability during long-time periods. The sampling might be done at the same month every year or using average annual values. Therefore they are suitable for monitoring chronic effluents, as river discharges or sewage, but not for one-time discharges.

8. Fucoid macroalgae can be used for monitoring nitrogen sources over long spatial scales (km), as their tissue $\delta^{15}N$ integrates the short time scale variability in nitrogen sources. In addition, they are reliable indicators of local nitrogen impacts when comparing $\delta^{15}N$ values with those of reference sites.

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Summary (Resumen)

I. Introducción general

Las costas, y en especial los estuarios, se han convertido en los depósitos finales de contaminantes de naturaleza industrial, urbana, agrícola, piscícola, etc. Se ha sugerido que la utilización de la relación de isótopos estables de nitrógeno ($\delta^{15}N$) es una herramienta prometedora como marcador de la presencia de fuentes de nitrógeno y del posible riesgo de eutrofización en sistemas costeros (McClelland y Valiela 1998). El empleo de la $\delta^{15}N$ se basa en que el nitrógeno tiene dos isótopos estables, uno ligero, ^{14}N, y otro más pesado, ^{15}N, en una proporción constante en la atmósfera de un 99.635 y un 0.365% respectivamente (Nier 1950). Esta proporción varía según las distintas rutas metabólicas que siga una molécula, pues el uso preferente del isótopo más ligero (fraccionamiento) de algunas reacciones del ciclo del nitrógeno produce distintas combinaciones de isótopos en los compuestos que reaccionan y en los productos de la reacción. La composición isotópica de los distintos compuestos se expresa en referencia al contenido isotópico del N_2 atmosférico, utilizado como estándar internacional. Debido a que las diferencias isotópicas entre compuestos son pequeñas, la relación se expresa como la variación respecto al estándar (δ) en partes por mil (‰):

$$\delta^{15}N\ (‰) = (R_{muestra} - R_{estándar} - 1) * 10^3$$

en donde R es $(^{14}N+^{15}N)/(^{14}N+^{14}N)$. Por lo que según esta notación, la $\delta^{15}N$ del N_2 atmosférico es 0‰.

En la década de 1950 se observó por primera vez la existencia de variaciones significativas en la abundancia natural de ^{15}N en distintos organismos marinos (Hoering 1955). Pero los primeros estudios centrados en la abundancia isotópica del nitrógeno en estos sistemas no se llevaron a cabo hasta mediados de 1960 (Miyake y Wada 1967, Wada et al. 1975, Wada y Hattori 1976, Minagawa y Wada 1984). Su uso como indicador de fuentes de nitrógeno en sistemas marinos no se generalizó hasta la década de 1990 (McClelland y Valiela 1998, Costanzo et al. 2001, Rogers 2003).

La utilización de la $\delta^{15}N$ como trazador de la contaminación marina se basa en que las distintas fuentes de nitrógeno antropogénico alteran la $\delta^{15}N$ de las matrices

bióticas y abióticas marinas (agua y sedimento) (Voβ y Struck 1997, McClelland y Valiela 1998). Así, actualmente, la $\delta^{15}N$ de los vertidos agrícolas suele estar empobrecida respecto a la $\delta^{15}N$ del medio marino. Esto se debe a que el nitrato y amonio de los fertilizantes derivan de la fijación industrial de N_2 atmosférico, y este proceso sufre un bajo fraccionamiento por lo que los productos resultantes tienen valores de $\delta^{15}N$ cercanos a 0‰ (Heaton 1986). Por el contrario, el componente dominante del nitrógeno total en vertidos urbanos o granjas de acuicultura tiene, en general, una $\delta^{15}N$ significativamente mayor que la $\delta^{15}N$ del nitrógeno inorgánico marino como resultado de la volatilización y transformación microbiana del nitrógeno en disolución (Heaton 1986, Van Dover et al. 1992). Por ello, la diferencia de la $\delta^{15}N$ de los aportes terrestres respecto a los ecosistemas costeros podría permitir cuantificar los aportes de nitrógeno de origen antropogénico al mar (McClelland y Valiela 1998).

Diversos estudios han utilizado las macroalgas para detectar la presencia y cuantificar el impacto de diversas fuentes de nitrógeno antropogénico (McClelland y Valiela 1998, Gartner et al. 2002). Las ventajas de estos organismos es que son fáciles de identificar, muestrear y procesar. Se considera que las macroalgas acumulan las sustancias disueltas en el agua en proporción a su biodisponibilidad en el medio. Puesto que no se han descrito procesos de fraccionamiento en la absorción de nitrógeno en algunas especies de macroalgas estudiadas (Naldi y Wheeler 2002, Cohen y Fong 2005) y son sésiles, se supone que integraran las variaciones de las fuentes de nitrógeno de una localidad durante determinados periodos de tiempo.

Las macroalgas de la familia Fucaceae han sido ampliamente utilizadas como biomonitores de distintos contaminantes desde hace décadas, entre ellas las macroalgas del género *Fucus* y *Ascophyllum nodosum*. La principal ventaja de estas especies es que presentan crecimiento apical, lo que permitiría delimitar los períodos en los que las distintas partes del talo estuvieron expuestas a distintas condiciones ambientales. Esta característica ha sido explotada de dos formas: mediante el análisis de los ápices en crecimiento para estudiar las condiciones recientes (Deutsch y Voss 2006); o mediante la división del talo en diversos segmentos para estudiar las condiciones pasadas (Savage y Elmgren 2004, Carballeira et al. 2014). Estos estudios han requerido del establecimiento de diversas asunciones relacionadas con la ecología, fisiología y procesos de fraccionamiento de macroalgas.

Otra de las ventajas de estas especies son su amplia distribución geográfica y su tolerancia a un amplio rango de condiciones abióticas, lo que permite realizar estudios a gran escala espacial. También son algas perennes, con persistencia en el sustrato durante varios años, posibilitando los estudios de variabilidad de los impactos. Debido a estas características también están sujetas a una gran variabilidad espacial, anual e interanual en la influencia de las fuentes de nitrógeno y de los factores abióticos, que en última instancia afectarán a los valores isotópicos de los tejidos de las macroalgas. A pesar del número de estudios que han empleado la medida de la $\delta^{15}N$ en macroalgas, los trabajos centrados en examinar las fuentes de variabilidad que afectan a estos valores son escasos, especialmente en macroalgas pardas (Umezawa et al. 2007, García-Sanz 2009). Sin embargo, para interpretar correctamente las relaciones entre la composición isotópica de las macroalgas y las presiones antropogénicas es preciso conocer bien las fuentes de variabilidad de la $\delta^{15}N$ bajo distintas circunstancias. Para ello es necesario llevar a cabo medidas simultáneas de isótopos de nitrógeno en macroalgas y agua. El fin es establecer las bases para la utilización de dichas macroalgas intermareales como biomonitoras de la incorporación de nitrógeno antropogénico en los ecosistemas litorales.

II. Hipótesis y objetivos generales

Teniendo en cuenta los conocimientos hasta la fecha se estableció la siguiente hipótesis:

- La proporción de isótopos estables de nitrógeno en los tejidos estructurales de macroalgas de vida larga refleja la distinta utilización de fuentes naturales o antropogénicas de nitrógeno en periodos de tiempo largos (meses, años).

Con el fin de validar esta hipótesis se estableció el siguiente objetivo principal:

- Determinar la relación entre la abundancia natural de isótopos estables de nitrógeno en macroalgas de vida larga y el origen de las fuentes de nitrógeno disponibles.

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Este objetivo se subdividió en los siguientes objetivos parciales:

1. Determinar la variabilidad geográfica de la composición isotópica de las macroalgas *A. nodosum* y *Fucus* spp. en función de factores naturales (ej. afloramiento) o antropogénicos (ej. urbanización).

2. Determinar la permanencia de la composición isotópica en distintas partes del talo de las macroalgas

3. Determinar las tasas de crecimiento de *A. nodosum* y *Fucus vesiculosus* para su posible aplicación en estudios retrospectivos de aportes de nitrógeno.

4. Cuantificar el impacto del nitrógeno antropogénico en macroalgas litorales.

III. Principales objetivos, resultados y conclusiones de cada capítulo

- **Isótopos estables de nitrógeno en macroalgas costeras: Variabilidad geográfica y antropogénica**

En la costa, aparte de las fuentes de nitrógeno antropogénico, varios procesos naturales contribuyen al enriquecimiento isotópico del nitrógeno disuelto. La costa del NO de España se caracteriza por la presencia de núcleos de población humana de tamaño variable y por la presencia de fenómenos de afloramiento durante los meses de primavera y verano. El objetivo de este capítulo fue determinar la importancia relativa de las fuentes de nitrógeno antropogénico y naturales en macroalgas pardas del intermareal a lo largo de esta área geográfica. Para ello se determinó la $\delta^{15}N$ en dos especies de macroalgas (*A. nodosum* y *F. vesiculosus*), así como en nitrato y amonio muestreado simultáneamente con las algas en las inmediaciones de poblaciones urbanas de distinto tamaño (~240 a ~246.000 habitantes) a lo largo de la costa del NO España (de Asturias al sur de Galicia).

Ambas especies de macroalgas y ambas fuentes de nitrógeno mostraron un enriquecimiento isotópico similar en determinadas localidades, sin embargo no se pudo establecer una relación simple entre la $\delta^{15}N$ de las algas y las concentraciones ni la señal isotópica del nitrógeno inorgánico. Estos resultados sugieren una alta

variabilidad de los aportes de nitrógeno inorgánico, de forma que no siempre dejan su huella isotópica en macroalgas, al menos en cortos intervalos de tiempo.

Por otro lado se observó una disminución lineal significativa de la $\delta^{15}N$ de *A. nodosum* y *F. vesiculosus* a lo largo de la costa. Esta disminución de los valores isotópicos puede estar relacionada con una menor intensidad del afloramiento que se observa de sur a norte en esta zona. Además de esta variabilidad geográfica, la influencia de las fuentes de nitrógeno antropogénico en macroalgas se evidencian por valores de $\delta^{15}N$ más elevados en rías y estuarios, en comparación con los de las zonas costeras más expuestas, y en áreas con poblaciones mayores de 15.000 habitantes.

Estos resultados indican que, al contrario de lo observado en otros estudios, los valores de $\delta^{15}N$ en macroalgas no están directamente relacionados con las concentraciones de nitrógeno inorgánico o con el tamaño de la población humana, sino que depende de otros factores como los fenómenos de afloramiento o factores locales, como la eficiencia de los sistemas de tratamiento de residuos.

- **Ecología de *F. vesiculosus* (Phaeophyceae) en su límite sur de distribución: Crecimiento y producción en los estadios tempranos de desarrollo**

Antes de utilizar las medidas de la $\delta^{15}N$ en distintos segmentos del talo de las macroalgas para inferir la variación temporal del impacto de distintas fuentes de nitrógeno, es necesario conocer el momento en que cada segmento estaba en fase activa de crecimiento y, por tanto, de absorción de nitrógeno del medio. Para ello es preciso disponer de métodos para determinar la edad del talo empleando curvas de crecimiento en longitud u otros marcadores. En el caso de *F. vesiculosu*s, ante la ausencia de estudios específicos en la zona de estudio, se determinó su crecimiento analizando poblaciones naturales de Galicia, que constituye el límite meridional de distribución de diversas especies de macroalgas pardas.

Se seleccionaron dos poblaciones de esta macroalga, una en una zona estuárica y otra semi-expuesta de la Ría de A Coruña. Para ello se siguió, durante 17 meses, la repoblación de tres áreas de muestreo (50x50 cm) denudadas experimentalmente en la zona del intermareal donde esta especie es dominante. El objetivo fue cuantificar las tasas de crecimiento, supervivencia, reproducción y producción en estas poblaciones después del raspado.

Durante el tiempo de muestreo se detectaron tres cohortes diferentes, entre las que se observaron diferencias en términos de crecimiento, reproducción y supervivencia. Estas diferencias pueden deberse a dos razones, bien a las distintas estaciones del año en las que se establecieron las cohortes o bien a la presencia de una cobertura de individuos previamente implantados pertenecientes a la segunda y tercera cohorte, que facilitaría la supervivencia y crecimiento de los nuevos reclutas. Así, el crecimiento de las cohortes reclutadas en otoño fue mayor que el de las cohortes reclutadas con posterioridad. En todos los casos el crecimiento individual estuvo representado por una función logística. Las mayores tasas de crecimiento en longitud se observaron durante los primeros seis meses de vida, y la longitud máxima se alcanzó cuando los individuos tenían un año de vida.

De la misma manera, la producción fue máxima para la primera cohorte, reclutada en otoño, incluso a pesar de presentar la tasa de supervivencia más baja, debido al rápido crecimiento de los supervivientes durante la primavera y el verano. La protección frente al oleaje de la zona estuárica pudo favorecer una mayor producción y mayores valores de biomasa en esta población que la de la zona semi-expuesta. Por el contrario, la tasa de renovación de biomasa fue mayor en la zona semi-expuesta. En ambas poblaciones, se observaron individuos con receptáculos durante todo el año, observándose el porcentaje máximo en primavera y verano. Además de proporcionar curvas de crecimiento para estimar la edad de cada segmento del talo, los resultados obtenidos sugieren que ambas poblaciones estudiadas son sensibles al daño mecánico, esta perturbación causaría una pérdida de biomasa y disminución de la producción, a pesar del rápido crecimiento inicial de los nuevos reclutas.

- **Crecimiento y producción de nuevos reclutas e individuos adultos de *A. nodosum* pertenecientes a una población no explotada en su límite sur de distribución (Galicia, NO España)**

De forma paralela al estudio del capítulo anterior, el objetivo de este capítulo fue estudiar el crecimiento de *A. nodosum* en Galicia, y de forma complementaria estudiar las tasas de reclutamiento, supervivencia y producción de biomasa de individuos menores de 2 años de edad. Para ello se monitorizó la repoblación de tres áreas de muestreo (50x50 cm) denudadas experimentalmente en la zona del intermareal donde esta especie es dominante. El crecimiento y la supervivencia fueron descritos como funciones continuas y no lineales que se aplicaron a la población y se utilizaron posteriormente para hacer estimaciones de su producción a distintas edades.

Las poblaciones de esta macroalga se caracterizan por estar principalmente formadas por individuos adultos y pocos reclutas, debido a que presentan un bajo éxito reproductivo y un crecimiento lento. En estas poblaciones la permanencia de los individuos adultos es de vital importancia para el mantenimiento de la población, pudiendo llegar a encontrar especímenes de hasta 14 años. Por ello, la demografía de los individuos mayores de dos años de edad se estudió en la población original (previa al raspado) después de la estimación de la edad de cada individuo basándose en el número de vesículas a lo largo del talo y en su longitud total (Niell 1979). Con el fin de determinar las distintas tasas de crecimiento de esta especie a escala local, se estudiaron diversas poblaciones adultas de otras localidades a lo largo de la costa de Galicia siguiendo el mismo método.

Se observó que el reclutamiento de *A. nodosum* en superficies previamente denudadas necesita una cobertura previa de otra macroalga (como *F. vesiculosus*), lo que crea un ambiente más húmedo y protegido para la supervivencia de los reclutas. Esto explicaría que durante los 26 meses que duró el estudio, la única cohorte detectada fue observada después del incremento en el número de individuos de *F. vesiculosus*.

Las bajas tasas de producción estimadas (2,033 g m^{-2} durante un período de 10 años) y el bajo reclutamiento indican que la población estudiada presenta una alta sensibilidad a la denudación. Sin embargo, la variabilidad observada entre las curvas estimadas de crecimiento de las diferentes poblaciones a lo largo de esta zona sur de distribución sugiere la existencia de un gran potencial de adaptación a las condiciones locales, que pueden llegar a superar los cambios ambientales a escala regional.

- **Evaluación experimental de la utilización de las macroalgas *A. nodosum* y *F. vesiculosus* para el seguimiento de fuentes de nitrógeno a distintas escalas de tiempo mediante la utilización de isótopos estables**

Algunas de las suposiciones establecidas para poder utilizar la composición isotópica de macroalgas pardas como biomonitores de vertidos antropogénicos de nitrógeno están todavía sin comprobar. Por ello, en este estudio se llevaron a cabo varios experimentos en laboratorio con las especies *A. nodosum* y *F. vesiculosus* con el fin de determinar algunos aspectos de la dinámica interna de los isótopos de

nitrógeno de estas macroalgas. El objetivo del primer experimento era determinar el tiempo de equilibrio de la $\delta^{15}N$ en los ápices en crecimiento y en las partes más antiguas del talo de las macroalgas. Para ello se incubaron ambas especies de macroalgas en agua con distintos orígenes, de una zona de marismas, de una zona con influencia antropogénica y de una zona con influencia oceánica. El objetivo del segundo experimento era detectar la posible existencia de transporte de nitrógeno y comprobar la capacidad de absorción de nitrato de las distintas partes del talo de las macroalgas. Para ello se utilizaron experimentos con agua enriquecida en ^{15}N.

Los resultados indican que el tiempo necesario para alcanzar el equilibrio de la zona apical con el agua es de 15 días para *F. vesiculosus* y de hasta 6 meses para *A. nodosum*. Las tasas de absorción más altas fueron observadas en los ápices en crecimiento, pero las partes más antiguas del fronde de ambas especies conservan, aunque en menor medida, la capacidad de incorporar nitrógeno. No se observaron evidencias de transporte de nitrógeno a lo largo del talo, tanto del ápice hacía el segmento basal del fronde o viceversa.

Estos resultados muestran que los ápices en crecimiento de estas macroalgas pueden ser utilizados para el seguimiento de vertidos de nitrógeno a escalas de tiempo de semanas (*F. vesiculosus*) a meses (*A. nodosum*). Aunque no se detectó transporte de nitrógeno a lo largo del talo, debido al intercambio cuantificable de nitrógeno entre las distintas partes del fronde y el agua no es recomendable el uso de las partes que no están en crecimiento apical para hacer estudios retrospectivos.

- **Variabilidad de la $\delta^{15}N$ de algas pardas intermareales a lo largo de un gradiente de salinidad: Impacto diferencial de las fuentes de nitrógeno**

La variabilidad de la $\delta^{15}N$ en macroalgas bentónicas fue estudiada a lo largo del sistema ría-estuario de A Coruña. El objetivo era determinar si la variabilidad de $\delta^{15}N$ en estas macroalgas era debida al impacto diferencial de las fuentes de nitrógeno o a factores intrínsecos de los individuos. Para ello se analizó la variabilidad temporal (mensual e interanual) y espacial (hasta 10 km) de la $\delta^{15}N$ en la macroalga *A. nodosum* y en tres especies del género *Fucus* (*F. serratus*, *F. spiralis* y *F. vesiculosus*). De forma paralela, se estudiaron las concentraciones de nitrato y amonio, y la $\delta^{15}N$ del nitrógeno inorgánico disuelto (DIN), junto con otros factores abióticos (salinidad y

temperatura) con el fin de detectar las posibles fuentes de variabilidad de la $\delta^{15}N$ en macroalgas.

La variabilidad estacional se estudió en una zona estuárica y otra semi-expuesta. En ambas localidades la $\delta^{15}N$ de las macroalgas fue menor que en el agua. Mientras que la señal isotópica de *F. vesiculosus* estaba correlacionada con la salinidad, la de *A. nodosum* no estaba correlacionada con ninguna variable medida en el agua. La $\delta^{15}N$ de ambas especies de macroalgas también mostró diferentes patrones en la diferencia interanual en estas mismas localidades. Mientras que la $\delta^{15}N$ de *A. nodosum* disminuyó desde 2010 hasta la actualidad, en *F. vesiculosus* se mostró estable o incluso aumentó en la localidad más expuesta.

En cuanto a la variabilidad espacial, se observó que las concentraciones de nutrientes y la $\delta^{15}N$ en macroalgas decrecían desde la zona estuárica hasta la zona de mayor influencia marina. Esto sugiere que, en este sistema, la influencia de las fuentes de nitrógeno antropogénico está principalmente limitada al estuario.

Los resultados obtenidos apoyan la utilización de la $\delta^{15}N$ de las macroalgas estudiadas para determinar el impacto de las distintas fuentes de nitrógeno integradas en un período largo de tiempo (meses), pues integran las pequeñas variaciones observadas en el agua. Sin embargo no servirían para detectar impactos puntuales en el tiempo, puesto que la señal isotópica en macroalgas no varía de la misma forma que la del DIN.

Los resultados de este estudio ponen de manifiesto la existencia de otras fuentes de variabilidad, aparte de las fuentes de DIN. Los valores de $\delta^{15}N$ en macroalgas fueron, en general, mayores que la señal isotópica del DIN a las escalas temporales y espaciales consideradas. Esto determinaría que la composición isotópica de las macroalgas está afectada por procesos de fraccionamiento durante la absorción, la asimilación o la liberación del nitrógeno de sus tejidos.

IV. Discusión general

Los trabajos que engloban esta tesis contribuyen a tener un mayor conocimiento de la variabilidad isotópica en las macroalgas estudiadas, aplicable para su utilización

como biomonitores. Los resultados obtenidos pueden ser aplicados tanto en estudios con algas nativas como con transplantes en el campo o laboratorio.

Estas macroalgas tienen como ventaja su gran extensión geográfica, lo que permite hacer estudios a escala regional. Para que esto sea posible hay que considerar la forma en la que influyen las distintas fuentes de nitrógeno naturales, pues pueden influir en los niveles de fondo establecidos. El gradiente observado en la $\delta^{15}N$ en *A. nodosum* y *F. vesiculosus* de la zona de afloramiento estudiada sugiere una influencia de estos procesos geográficos. Esto afecta directamente a la interpretación de resultados a una escala regional. Después de tener en cuenta la variabilidad geográfica, la $\delta^{15}N$ de las macroalgas permitió diferenciar el impacto de poblaciones urbanas de distintos tamaños. La variabilidad de los valores obtenidos, especialmente en las zonas influidas por poblaciones de pequeño tamaño, sugiere la existencia de distintos procesos de depuración o eficiencia variable de estos sistemas. Comparativamente, la eficiencia de los tratamientos de depuración de aguas residuales en grandes núcleos urbanos resultaría mucho más homogénea.

La principal ventaja que presentan estas macroalgas frente a otras especies es la posibilidad de su utilización para estudios retrospectivos de contaminación antropogénica (Savage y Elmgren 2004, Carballeira et al. 2014). Al ser posible datar el tiempo en que creció cada sección del talo se podrían obtener series temporales de $\delta^{15}N$ a partir de ejemplares muestreados en una solo ocasión. Con el fin de utilizar de forma fiable estas especies como biomonitores retrospectivos se procedió a estudiar dos aspectos clave, i) estimar el crecimiento de estas macroalgas con el fin de poder relacionar determinados segmentos con determinados tiempos de exposición, ii) verificar si la fisiología de las macroalgas podría interferir en la determinación de la $\delta^{15}N$. Por una parte se obtuvieron las curvas de crecimiento de ambas especies lo que permite datar los segmentos del talo. Sin embargo, los experimentos llevados a cabo confirmaron que todas las partes del talo conservan la capacidad de absorber nitrato, por lo que, a pesar de no existir transporte de nitrógeno a lo largo del talo, los distintos valores de $\delta^{15}N$ resultan de la integración desde que ese segmento estaba en crecimiento hasta el momento de muestreo. Así el uso como biomonitor se restringe a los ápices, determinándose que la utilización del segmento apical de 1 cm de longitud proveería de información integrada de las fuentes de nitrógeno en los últimos 14 días en *F. vesiculosus* y 21 días en *A. nodosum*.

El estudio llevado a cabo en la Ría de A Coruña resalta la importancia de entender los factores locales para la correcta interpretación de la $\delta^{15}N$ en macroalgas. A pesar de una coincidencia zonal entre los valores de $\delta^{15}N$ en DIN y en macroalgas (que indican un mayor impacto antropogénico en el estuario), no es posible establecer una relación cuantitativa directa entre la señal isotópica en agua y macroalgas a lo largo del sistema ría-estuario estudiado. Teniendo en cuenta el tiempo de integración de estas macroalgas, se puede determinar que ambas matrices simplemente representan distintas escalas de tiempo.

El análisis simultáneo de la $\delta^{15}N$ en macroalgas y agua pone de manifiesto la importancia del metabolismo de las macroalgas, que parece más importante incluso que la variabilidad espacial o temporal observada. Aunque el fraccionamiento durante la absorción de nitrógeno ha sido estudiado en algunas especies (Cohen y Fong 2005, García-Sanz 2009), poco se sabe del fraccionamiento neto resultante de todos los procesos metabólicos que sufre el nitrógeno desde su absorción hasta su excreción en las macroalgas. Por tanto las investigaciones futuras deberían centrarse en determinar los procesos de fraccionamiento que afectan a la $\delta^{15}N$ de las macroalgas con el fin de optimizar el uso de estas especies como biomonitores de nitrógeno mediante el análisis de $\delta^{15}N$.

V. Conclusiones generales

1. Los valores de $\delta^{15}N$ de *A. nodosum* y *F. vesiculosus* están influenciados significativamente por la huella isotópica de las fuentes antropogénicas (aguas residuales) y naturales (afloramiento costero). Para una correcta interpretación de los resultados en un área geográfica en la que tengan lugar fenómenos de afloramiento se requiere un conocimiento previo de la influencia de este fenómeno en la $\delta^{15}N$ de cada localidad.

2. La $\delta^{15}N$ de *A. nodosum* y *F. vesiculosus* aumenta con el tamaño poblacional de los núcleos urbanos <15.000 habitantes pero con una alta variabilidad asociada, mientras que las poblaciones de mayor tamaño no se pueden relacionar con ningún rango de valores de $\delta^{15}N$. Esto sugiere la existencia de diferencias en la eficiencia de retirada de nitrógeno de las aguas residuales de las ciudades de pequeño tamaño o bien diferencias en los tratamientos de estas aguas.

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3. Posteriormente a la denudación experimental, las poblaciones de *F. vesiculosus* fueron reclutadas en tres cohortes diferentes durante los 17 meses que duró el estudio. Las curvas de crecimiento obtenidas siguen una ecuación logística y se observaron diferentes tasas de crecimiento entre las cohortes establecidas en diferentes tiempos a lo largo del año, y entre las poblaciones estudiadas (semi-expuesta y estuárica). En general, un fragmento de 1 cm del ápice en crecimiento representaría el crecimiento durante los 14 días previos de un individuo de una población estuárica.

4. El reclutamiento de *A. nodosum* en los cuadrantes denudados experimentalmente fue un proceso lento y solo se observó una cohorte en los 26 meses que duró el estudio. Con respecto a las poblaciones adultas, se encontraron diferencias entre las curvas de crecimiento estimadas en las diferentes localidades, lo que destaca la importancia de los factores locales en el crecimiento de esta especie. En general, en una población estuárica, un fragmento de 1 cm del ápice en crecimiento representaría el crecimiento del individuo de los 21 días previos.

5. Los fragmento basales y partes antiguas de los frondes de *A. nodosum* y *F. vesiculosus* no se deberían de utilizar en estudios retrospectivos de monitorización con isótopos estables puesto que mantienen la capacidad de absorber nitrógeno. Se recomienda el uso exclusivo de los ápices aprovechando el crecimiento apical de estas especies y que no se observó transporte a lo largo del talo.

6. Los distintos valores de $\delta^{15}N$ observados en macroalgas no se pueden relacionar directamente con la huella isotópica del nitrógeno disuelto tanto a escala regional como local. Esto es debido a que *A. nodosum* integra la variabilidad del nitrógeno disuelto durante 6 meses si el individuo estuviera todo el tiempo sumergido; mientras que *F. vesiculosus* integra la variabilidad del nitrógeno disuelto durante 15 días. Esto resulta especialmente importante en los estudios con transplantes o en laboratorio.

7. El uso de la $\delta^{15}N$ en macroalgas para estudiar la variabilidad interanual es apropiada puesto que son especies perennes e integran la variabilidad del nitrógeno disuelto durante largos períodos de tiempo. El muestreo debe ser

realizado el mismo mes todos los años o utilizando valores medios anuales. Por lo que las macroalgas estudiadas son adecuadas para la monitorización de vertidos crónicos, como ríos o aguas residuales, pero no para vertidos puntuales.

8. Las macroalgas fucales pueden ser utilizadas para monitorizar fuentes de nitrógeno a escalas espaciales de kilómetros, puesto que la $\delta^{15}N$ de sus tejidos integra las pequeñas variaciones del nitrógeno disuelto. Además, son biomonitores fiables de impactos a escala local cuando sus valores se comparan con los de lugares de referencia.

VI. Referencias

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The greatest glory in living lies not in never falling, but in rising every time you fall

(Nelson Mandela)

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